# Endocrine disrupting pesticides in environmental samples


## José Luis Vera

Doutoramento em Química Sustentável

Departamento de Química e Bioquímica

2014

### Orientador

Doutor José Alberto Nunes Ferreira Gomes, Professor Catedrático, Faculdade de Ciências, Universidade do Porto

### Co-orientador

Doutora Cristina Delerue-Matos, Professor Coordenador, REQUIMTE - Instituto Superior de Engenharia, Instituto Politécnico do Porto

Doutora Valentina Fernandes Domingues, Professor Coordenador, REQUIMTE - Instituto Superior de Engenharia, Instituto Politécnico do Porto

# Tese de Doutoramento

# Endocrine disrupting pesticides in environmental samples

***José Luis Vera***

***Dissertação para obtenção do Grau de Doutor em Química Sustentável, apresentada à Faculdade de Ciências da Universidade do Porto***

**Orientação**

Professor Doutor José Alberto Nunes Ferreira Gomes

**Co-Orientação**

Professora Doutora Cristina Delerue-Matos

Professora Doutora Valentina Fernandes Domingues

Porto

Janeiro 2014

**A realizaçao deste trabalho foi possível graças à concessão de uma Bolsa de Doutoramento pelo Programa Erasmus Mundus Ação 2, Vertente 1 – Projeto MUNDUS 17 – lote 17 – Brasil, Paraguai e Uruguai – financiado pela Comissão Europeia através da EACEA (The Education Audiovisual and Culture Executive Agency).**

**Os estudos apresentados nesta dissertação foram realizados no Laboratório Grupo de Reaçao e Análise Química (GRAQ) do Instituto Superior de Engenharia do Porto do Instituo Politécnico do Porto.**

# Agradecimentos

O meu profundo obrigado à família, aos professores, aos amigos, aos colegas, às instituições e aos serviços que contribuíram para que o presente trabalho de doutoramento fosse uma realidade.

Agradeço:

Aos responsáveis da Facultad de Ciencias Exactas y Naturales (FaCEN) e à Dirección de Relaciones Internacionales da Rectoria da Universidad Nacional de Asunción pela adesão ao projeto MUNDUS 17 (Programa Erasmus Mundus Ação 2, Vertente 1 – Projeto MUNDUS 17 – lote 17 – Brasil, Paraguai e Uruguai – financiado pela Comissão Europeia através da EACEA (The Education Audiovisual and Culture Executive Agency)), para obtenção da bolsa de doutoramento, responsável pelo suporte financeiro deste trabalho de investigação e de uma bolsa do GRAQ/ Instituto Superior de Engenharia do Porto concedida pelo Instituto Politécnico do Porto ao abrigo do programa PAPRE (GRAQ/2013/10) e pelo que estou muitíssimo agradecido pela oportunidade que me concederam e que enriqueceu a minha formação profissional e pessoal.

À instituição de acolhimento (Universidade do Porto), principalmente à Marília Cunha, Ana Castro Paiva, Miu Castro Paiva e Dra. Paula Marques da equipa de Gestão com quem estive em contacto desde o começo da mobilidade do Erasmus Mundus 17; elas ajudaram ao esclarecimento das dúvidas relacionadas e da ajuda em toda a gestão administrativa e a resolução das dificuldades que foram surgindo durante o percurso da mobilidade.

Ao Professor Doutor José Ferreira Gomes por ter aceitado ser meu orientador e me ter encaminhado para o laboratório de investigação GRAQ, para a realização da minha tese de doutoramento e ainda pela ajuda na resolução de alguns problemas relacionados com a documentação do doutoramento.

À Professora Doutora Cristina Delerue Matos, quero manifestar o meu agradecimento, pela oportunidade de me inserir no seu grupo de investigação no Laboratório GRAQ/REQUIMTE. Realço a excelente pessoa que é, a quem admiro pela sua forma de lidar com todas as situações do dia-a-dia, pelas palavras de carinho e de amizade, sempre disposta a fazer o impossível para que todos consigam atingir os seus objetivos.

À Professora Doutora Valentina Domingues por me ter orientado e acompanhado ao longo destes três anos, ao qual manifesto o meu profundo agradecimento pela orientação, disponibilidade, apoio, compreensão e carinho. Estando sempre presente em todos os momentos e dificuldades neste trabalho, na ajuda das publicações dos artigos, dos posters, e da presente tese.

À Doutora Virgínia Fernandes pelos ensinamentos, ideias, soluções, pela amizade e pelo carinho. O meu agradecimento sincero por a ter conhecido e por fazer parte, sem dúvida alguma, dos melhores momentos desta etapa da minha vida.

Ao J. M. Costa e Doutora Catarina Mansilha pela colaboração com o Laboratório Ricardo Jorge, na colheita de amostragem da Ria de Aveiro.

Ao Dr. Luís Silva, pela sua disponibilidade e ajuda nos cálculos estatísticos na otimização da metodologia de espectrometria de massa.

Aos meus colegas e amigos de trabalho, Paula, Luísa, Isabel, Idalina, Teresa, Marta, António, Ivo, Eng. Isabel, Filipa, Rita, Susana, Alexandra, Laurens, Ruth e a cada um que faz parte do grupo

GRAQ que, em maior ou menor medida, tornaram-se parte deste trabalho e do dia-a-dia como uma equipa.

Ao Doutor Viswanathan com quem aprendi que o conhecimento é poder, que o trabalho duro tem os seus frutos, e que lutar faz parte da vida. Ao Francisco Wirley, Túlio e Thiago por serem amigos e irmãos na fé e pelos bons momentos que passamos juntos.

Às pessoas da minha Igreja, em Portugal e no Paraguai, por estarem presentes nos momentos difíceis e de por me darem tão gratos momentos, dos quais especialmente queria agradecer ao Pastor António Figueira e à sua família por me receberem em Portugal.

Ao amigo e professor de inglês Jeffrey Darling, pela imensa ajuda na correção dos textos escritos em inglês e artigos relacionados com a tese.

Ao Gigi e sua esposa Rodica, por me terem acolhido na sua casa fazendo-me sentir como parte da família.

Um agradecimento com carinho à minha namorada Isabel, sua mãe Manuela e sua irmã Lídia e marido David pela amizade, pela ternura e pelo apoio.

À minha família, a quem amo muito, em especial os meus pais Feliciano e Justina, aos meus irmãos Gladys, Leticia, Luis, Manuel e Ulises, ao meu cunhado Victor e à minha avó Maximina. Todos eles têm sido o meu porto seguro em todas as etapas da minha vida.

E finalmente, para o Rei dos Reis que é o meu Senhor Jesus, minha esperança e meu refúgio, embora eu seja fraco, com Ele sou forte, foi quem me deu a vida e me ama de tal maneira que entregou a Sua vida por mim; a Ele agradeço por me ter trazido para realizar este doutoramento.

Muito obrigado!

# Resumo

Os pesticidas têm sido usados para impedir danos nas culturas. No entanto, são conhecidas as consequências nefastas do uso excessivo destes compostos na agricultura. Em Portugal, o uso de pesticidas na região Norte é intenso, tornando-o vulnerável à contaminação. Destacam-se as famílias de pesticidas organoclorados, triazinas, piretróides, organofosforados, etc.. Grande parte destes pesticidas tem vindo a ser considerados como desreguladores endócrinos ou como potenciais desreguladores, sendo por isso alvo de diversos estudos por parte da comunidade científica e técnica no que concerne à sua monitorização e avaliação dos efeitos nocivos para o Homem e ecossistemas.

Deste modo, esta tese focou-se principalmente na otimização de métodos de extração (extração em fase sólida (SPE), micro extração em fase sólida (SPME) e QuEChERS) e técnicas cromatográficas para análise de desreguladores endócrinos. O presente trabalho de investigação abrangeu o estudo de trinta e oito pesticidas e seus metabolitos: organoclorados (aldrin, DDT, p,p-DDD, o,p-DDE, HCB, dieldrin, isómeros de HCH, endosulfan, endrin, heptacloro, metoxicloro e metolacloro), triazinas (atrazina, desetil atrazina, terbutilazina, desetil terbutilazina e simazina), piretróides (bifentrina, cipermetrina, ciflutrina, cialotrina, deltametrina, fenvalerato, fenpropatrina e permetrina), organofosforados (diazinão e malatião), dicarboximida (iprodiona e vinclozolina), organofosfato (dimetoato), ácido/éster ariloxialcanoico (éster metílico de 2,4-D), tiocarbamato (EPTC), ureia (linurão), dinitroanilina (pendimetalina) e cloroacetamida (alacloro). Foi utilizada a cromatografia gasosa (CG) com espectrometria de massa (MS) na quantificação, identificação e confirmação da maior parte dos pesticidas em estudo. Na deteção por GC-MS/MS optou-se por uma abordagem estatística de otimização multivariável dos parâmetros instrumentais tendo sido o método aplicado na análise de pesticidas organoclorados, triazinas e piretróides, em águas. Relativamente às técnicas de extração foram utilizados SPE e SPME na extração de pesticidas nas águas de rios e estuários e a metodologia QuEChERS na extração dos pesticidas em sedimentos. Estas metodologias foram aplicadas a amostras de águas de rio e estuarinas recolhidas no período compreendido entre 2010 e 2012, cujos locais de amostragem se situavam na região norte de Portugal: Ria de Aveiro, Rio Douro, Rio Cávado, Rio Lima, Rio Minho, Rio Sousa, Rio Tâmega, Rio Leça, Rio Cabrum, Ribeira Moscoso, Rio Caima e Rio Ave. A metodologia SPME-GC-MS foi usada na monitorização de pesticidas em águas dos rios. Os resultados mostraram níveis de pesticidas mais elevados no Rio Douro com a concentração de 1800 ng / L (γ-HCH) e no Rio Cávado com a concentração de 760 ng / L (HCB). A metodologia SPE-GC-MS foi aplicada a amostras de água recolhidas na Ria de Aveiro, por ser uma zona de intensa agricultura. Foram quantificados catorze compostos, e os valores encontrados oscilaram na gama de

concentrações de 32 a 3446 ng / L. A aplicação da técnica de extração QuEChERS à análise de pesticidas em sedimentos em que a quantificação foi efetuada por GC-MS, os resultados evidenciaram a presença de desethyl atrazina, β-HCH, fenvalerato, com variação das concentrações entre 4.0 a 27.7 ng / g.

Termos chave: Pesticidas, Desreguladores endócrinos, GC-MS, GC-ECD, SPE, SPME, QuEChERS, Águas, Sedimentos

# Abstract

Humans have always used pesticides to prevent damage to their crops. However, it is not a secret that there are adverse consequences from excessive use of these compounds in agriculture. In Portugal, the use of pesticides in the northern region is frequent, making the region vulnerable to contamination. The most common pesticides come from the families of organochlorine, triazines, pyrethroids, and organophosphates. Most of these pesticides have been considered true endocrine disruptors, or have the potential to become so. Therefore, they are the subject of several studies by the scientific community in regards to monitoring adverse effects on humans and ecosystems. These studies focused primarily on the optimization of extraction methods (SPE, SPME, QuEChERS), and on chromatography for extraction and analysis of endocrine disrupters.

This present study includes the analysis of thirty-eight pesticides and metabolites: organochlorine (aldrin, DDT, p,p-DDD, o,p-DDE, HCB, dieldrin, HCH isomers, endosulfan, endrin, heptachlor, methoxychlor, and metolachlor), triazines (atrazine, desethyl atrazine, terbuthylazine, desethyl terbuthylazine, and simazine), pyrethroids (bifenthrin, cypermethrin, cyfluthrin, cyhalothrin, deltamethrin, fenvalerate, fenpropathrin, and permethrin), organophosphate (malathion and diazinon), dicarboximide (iprodione and vinciozolin), organophosphate (dimethoate), acid ariloxialcanoico ester (methyl ester of 2,4-D), thiocarbamate (EPTC), urea (linuron), dinitroaniline (pendimethalin), and chloroacetamide (alachlor). Gas chromatography (GC) coupled with mass spectrometry (MS) was used for identification, quantification and confirmation of most of the analysed pesticides. Concerning detection using GC-MS/MS, a statistical approach to the multivariable optimisation of instrumental parameters was the method applied in analysis of organochlorine, triazines, and pyrethroids. This analysis was done in water. SPE and SPME were the techniques used to extract pesticides from the waters of rivers and estuaries. The QuEChERS procedure was used for the extraction of pesticides in sediments. These methodologies were applied to samples of river and estuarine waters collected in the period between 2010 and 2012. The sampling sites were located in the northern region of Portugal, and included: Ria de Aveiro, Douro River, Cávado River, Lima River, Minho River, Sousa River, Tâmega River, Leça River, Cabrum River, Ribeira Moscoso, Caima River, and Ave River. The SPME-GC-MS method was used for the monitoring of pesticides in river water. The results showed higher levels of pesticides in the Douro River, with a concentration level of 1800 ng/L (δ-HCH), and in the Cávado River, with a concentration level of 760 ng/L (HCB). The SPE-GC-MS method was applied to water samples collected in the Ria de Aveiro, being a zone of intensive agriculture. Fourteen compounds were quantified, and the values were found varied in the concentration range of 32 to 3446 ng/L. The use of the QuEChERS technique in the analysis

of pesticides in sediments showed the presence of desethyl atrazine, β-HCH, and fenvalerate. Quantification was performed by using the GC.MS method, and showed concentration levels between 4.0 to 27.7 ng / g

Keywords: Pesticides, Endocrine Disruptor, GC- MS, SPE, SPME, QuEChERS, Water, Sediment

# Resumen

Los seres humanos han usado los pesticidas para impedir daños en sus cultivos. Sin embargo, son conocidas las consecuencias nefastas del uso excesivo de estos compuestos en la agricultura. En Portugal, el uso de pesticidas en la región norte es intenso, volviéndolo vulnerable a la contaminación. Se destacan los pesticidas organoclorados, triazinas, piretroides, organofosforados, ect.. Grande parte de estos pesticidas han venido a ser considerados disruptores endocrinos o como potenciales disruptores endocrinos, siendo por eso objetivo de varios estudios por parte de la comunidad científica y técnica en lo que concierne a su monitorización y evaluación de efectos nocivos para el hombre y ecosistemas. De este modo, esta tesis se centro principalmente en la optimización de métodos de extracción (extracción en fase sólida (SPE), micro extracción en fase sólida (SPME), y QuEChERS) y en la técnicas cromatográficas para la extracción y análisis de los disruptores endocrinos. El presente trabajo investigación abarco el estudio de treinta y ocho pesticidas y sus metabolitos: organoclorados (aldrina, DDT, p,p-DDD, o,p-DDE, HCB, dieldrina, isómeros del HCH, endosulfán, endrina, heptacloro metoxicloro y metolachlor), piretroides (bifentrina, cipermetrina, ciflutrina, cihalotrina, deltametrina, fenvalerato, fenpropatrin y permetrina) organofosforados (malatión y diazinón) dicarboximida (iprodiona y vinclozolina), organofosforados (dimetoato), éster de ácido / ariloxialcanoico (éster metílico de 2,4-D) tiocarbamato (EPTC), urea (linuron), dinitroanilina (pendimetalina) y cloroacetamida (alaclor). Fue utilizado la cromatografía gaseosa (CG) con espectrometría de masa (MS) en la cuantificación, identificación, y confirmación de la mayor parte de los pesticidas en estudio. En la detección por GC-MS/MS se optó por un abordaje estadístico de optimización multivariable de los parámetros instrumentales siendo el método aplicado en el análisis de pesticidas organoclorados, triazinas y piretroides, en las aguas. Relativamente a las técnicas de extracción, fueron utilizados SPE y SPME en la extracción de pesticidas en las aguas de ríos y estuarios y la metodología QuEChERS en la extracción de pesticidas en sedimentos. Estas metodologías fueron optimizadas y posteriormente aplicadas en las muestras de aguas de rio recogidas en el periodo comprendido entre 2010 y 2012, cuyo locales de muestreo se situan en la región norte de Portugal: Ria de Aveiro, Rio Douro, Rio Cávado, Rio Lima, Rio Minho, Rio Sousa, Rio Tâmega, Rio Leça, Rio Cabrum, Ribeira Moscoso, Rio Caima y Rio Ave. La metodología SPME-GC-MS fue usada en la monitorización de pesticidas en las aguas de ríos. Los resultados muestran los niveles de pesticidas más elevados en el Rio Douro con concentraciones de 1800 ng / L (γ-HCH) y en el Rio Cávado, con una concentración de 760 ng / L (HCB). La metodología SPE-GC-MS fue aplicada para las muestras de aguas recogidas en la Ria de Aveiro, por ser una zona de intensa agricultura. Fueron cuantificados catorce compuestos, y los valores encontrados

oscilan en una gama de concentraciones de 32 a 3446 ng / L. La aplicación de la técnica de extracción QuEChERS, para el análisis de pesticidas en sedimentos fue efectuado por GC-MS, los resultados evidencian la presencia de atrazina desetil, β-HCH, fenvalerato, con concentraciones entre 4.0 a 27.7 ng / g.

Términos clave: pesticidas, los disruptores endocrinos, GC-MS, GC-ECD, SPE, SPME, QuEChERS, Agua, Sedimentos

# List of Contents

# List of Figures

# List of Tables

# List of Abbreviations

| | |
|---|---|
| 2,4 D | dichlorophenoxyacetic acid |
| ASE | Accelerated Solvent Extraction |
| CG | Gas Crhomatography |
| DDD | Dichlorodiphenyldichloroethane |
| DDE | dichlorodiphenyldichloroethylene |
| DDT | dichlorodiphenyltrichloroethane |
| DI-SPME | direct immersion solid-phase micro- |
| DL | Decree law |
| DLLME | Dispersive Liquid-Liquid Microextraction |
| DSPE | Dispersive solid phase extraction |
| EC | European Commission |
| EDCs | Endocrine Disrupting compounds |
| EI | Electron Ionization |
| EPA | Environmental Protection Agency |
| EPAL | Empresa Portuguesa das Águas Livres |
| ERSAR | Entidade Reguladora dos Serviços de Águas e Resíduos |
| ET | Excitation Time |
| EtAc | Ethyl Acetate |
| EV | voltage |
| GC × GC-TOFMS | Orthogonal Chromatography-Time-of-the-flight mass analyzer |
| GC-ECD | Gas Crhomatography-Electron capture detector |
| GC–MS | Gas chromatography–mass spectrometry |
| GPC | Gel-Permeation Chromatographic |
| HCB | Hexachlorobenzene |
| HCH | Hexachlorocyclohexane |
| HPV | High Production Volume |
| IMW | Isolation Mass Window (wideband application) |
| INRB | Instituto Nacional de Recursos Biológicos |
| INE | Instituto Nacional de Estatistica |
| IT | isolation time |
| LC | Liquid-Liquid Extraction |
| LD | Liquid Desorption |
| LLE | Liquid-Liquid Extraction |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| MAE | Microwave Assisted Extraction |
| MeCN | Acetonitrile |
| MgSO4 | Magnesium sulfate |

| | |
|---|---|
| MRMs | Multiresidue Methods |
| MLLE | Micro Liquid-Liquid Extraction |
| Na2SO4 | Sodium sulfate |
| NaCl | Sodium chloride |
| not pers | non-persistent |
| OC | Organochlorines |
| ON | Organonitrogen |
| OP | Organophosphate |
| PA | pol(acrylate) |
| PAHs | Polycyclic Aromatic Hydrocarbons |
| PCBs | Polychlorinated Biphenyls |
| PDMS | poly (dimethylsiloxane) |
| pers | persistent |
| pers + | highly persistent |
| PLE | Pressurized Liquid Extraction |
| POPs | Persistent organic pollutants |
| PSA | Primary Secondary Amine |
| PTV | Programmed-temperature vaporiser |
| Q | factor Q |
| QSAR | Quantitative structure–activity relationship |
| QuEChERS | Quick, Easy, Cheap, Effective, Rugged, and Safe |
| SBSE | Stir Bar Sorptive Extraction |
| SFE | Supercritical Fluid Extraction |
| SIM | Single-Ion Monitoring |
| SPE | Solid Phase Extraction |
| SPME | Solid Phase Microextraction |
| SXE | Soxhlet Extraction |
| TD | Thermal Desorption |
| UAE | Ultrasound-Assisted Extraction |
| TSH | Thyroid-stimulating Hormone |
| ER | Estrogen Receptor |

# Organization, and structure of the thesis

The present thesis includes all the work developed in the scope of the doctoral project inserted in the PhD program in Sustainable Chemistry. The document is divided into six chapters that enclose four scientific articles. One of the chapters includes an adapted version of a published article, and others three include three articles that were submitted to international peer-reviewed journals. The objective of including published and submitted articles was that almost all of the work had already been critically analysed by different international reviewers, experts in the research field in which the work was developed, and selected according to the criteria of the journals in which they were published. For all the articles, their structures were maintained according to the journal guidelines in which they were published or submitted to, including the reference style. However, the table and figure numbers were adapted according to the chapter in which they were inserted.

The first chapter corresponds to an introduction in order to contextualize the developed work, followed by the description of the general and specific objectives. This part of the thesis includes a review of the state-of-the-art regarding the analytical methodologies for the identification and quantification of Endocrine Disrupting Pesticides in the environment, focusing on the most recent trends in sample preparation and chromatographic separation.

Chapter 2 corresponds to part of the work included in a paper published in the Journal of The American Society for Mass Spectrometry and describes the optimization of the mass spectrometric parameters for the determination of 11 pesticides, belonging to three different families, by gas chromatography (GC) with ion-trap tandem mass spectrometric detection (MS).

Chapter 3 describes the development of a Solid Phase Micro Extraction (SPME) technique followed by GC with mass spectrometric (MS) detection. The methodology was used to evaluate twenty pesticides in surface water, in particular twelve rivers located in the north of Portugal.

To monitor the presence of thirty five pesticides and/or their metabolites throughout the environment, another approach, based on Solid Phase Extraction (SPE) and GC-MS, is described in Chapter 4. The developed methodologies were applied to the analysis of surface water samples from Ria de Aveiro, near Aveiro.

In Chapter 5 another and more recent sample preparation procedure was developed: “Quick, Easy, Cheap, Effective, Rugged and Safe”, referred to as QuEChERS. This method has many advantages for solid samples and was therefore applied to assess the presence of twenty six pesticides and three metabolites in sediments of Ria de Aveiro.

Finally, in Chapter 6 the final concluding remarks will be presented. The main conclusions of the developed work were presented along the different chapters, so in the last chapter the highlights are described and the future perspectives are pointed out. The end of this dissertation is not the culmination of a project, but it intends to formulate a set of reflections, prospecting projects and future research activities.

# Objetives

During recent years the repeated monitoring of endocrine disrupting pesticides (EDPs) in a variety of matrices resulted in several advances related to sample preparation techniques, especially extraction procedures, and their chromatographic separation.

Despite the previous studies, it was considered important to explore new analytical methodologies for EDPs control in environmental samples. This required the development of analytical methodsbut also were environmentally less aggressive than traditional methods. In this scope, the present work stems from an investigation that was based on the following general objectives:

- To develop new analytical methodologies for EDPs determination in the environment using new approaches, emphasizing green processes;
- To assess environmental quality regarding EDPs;
- To evaluate the influence of agricultural activities on the quality of surface waters with respect to EDPs;
- To disseminate the results obtained in Portuguese case studies.

To achieve the general objectives, the experimental work was developed to comply with the following specific objectives:

- To develop solventless extraction techniques, such as SPME and SPE, in order to reduce the solvent volume and sample manipulation as well as the analysis time and, simultaneously, minimize the amount of sample required for each analysis;
- To develop a “Quick Easy Cheap Effective Rugged Safe” (QuEChERS) extraction method for EDPs quantification in sediments;
- To develop gas chromatographic techniques, with mass spectrometry (MS) detection, for the environmental control of EDPs;
- To apply the developed methodologies to the analysis of EDPs in environmental samples, namely surface waters, such as river and estuary waters, and sediments;
- To monitor EDPs in environmental samples from the northern region of Portugal.

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1 Introduction

## 1.1 State of the art of endocrine disruptors

Thousands of chemicals (organic and inorganic) of anthropological origin are currently found in the environment. These chemicals cause changes in human physiological functions and, consequently, health problems. One of the subjects that have received tremendous attention from scientific and regulatory communities worldwide is the issue of endocrine disruption. Numerous studies have been carried out concerning the possible harmful consequences of human and wildlife exposure. The endocrine disruptors, also called endocrine-active compounds, endocrine modulators, environmental hormones, hormone-related toxicants etc., are compounds which exhibit the potential of interfering with the endocrine system of humans and animals. This group of endocrine disruptors is included with a large and still increasing number of natural and anthropogenic agents with diverse chemical structures. Figure 1.1 illustrates the endocrine system formed by a set of glands, such as the thyroid, gonads, adrenal and pituitary glands. Figure 1.1 also shows the hormones they produce, such as thyroxine, oestrogen, testosterone and adrenaline. These hormones regulate the development, growth, reproduction, and behaviour of animals, including human beings [1]. In addition, numerous physiological processes are controlled by these hormones, which are delivered from their tissues of production to their target organs via the blood stream. Table 1.1 summarizes some relevant endocrine system components and hormones (mainly mammalian) of interest in connection with environmental endocrine modulators.

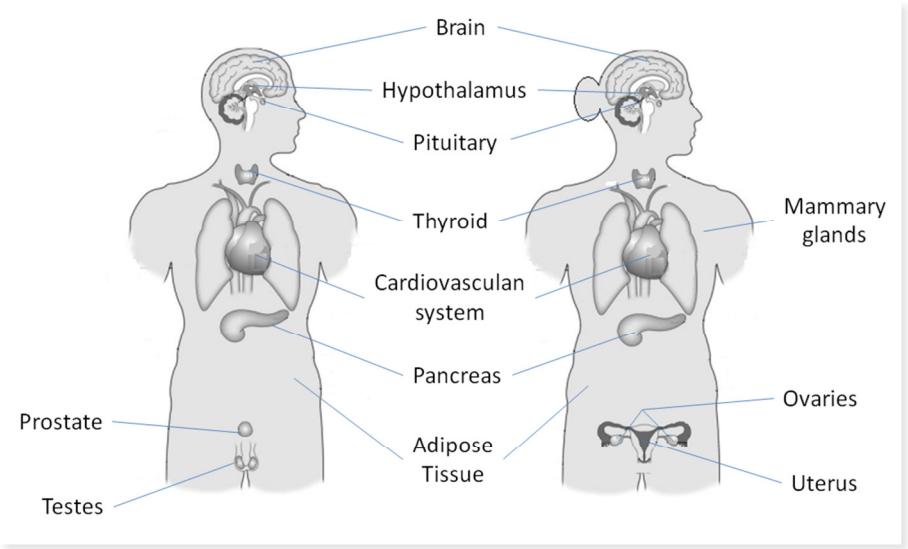



**Figure 1.1 Male and female endocrine system. Adapted from Diamanti-Kandarakis, et al. and Sharpe et al.[2,3].**

Hormones are signalling molecules which elicit responses in other parts of the body. An endocrine system is found in most animals, including mammals, non-mammalian vertebrates (such as birds, fish, amphibians, and reptiles), and in the invertebrates (such as snails and insects) [1].

By European Commission the endocrine disruptor is defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations". According to the Environmental Protection Agency (EPA); Endocrine disruptor is "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" [4].

**Table 1.1 Summary of some endocrine systems glands and hormones (mainly mammalian) of interest in connection with environmental endocrine modulators. Adapted from Louekari et al.[5].**

| System/gland | Important hormones produced | Important hormones received | Functions/regulation |
|---|---|---|---|
| Hypothalamus | GnRH (gonadotropin releasing hormone), CRH (corticotropin releasing hormone), TRH (thyrotropin releasing hormone) | - | Overall regulation of glands through pituitary/directly, e.g. in thyroid, and gonads |
| Pituitary | Gonadotrophic H (GTH)/gonadotropin (CG), Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), Prolactin (Prol), | GnRH | Gonad development, ovulation onset etc., sexual/reproductive functions, lactation (brain development), thyroid regulation, and regulation of energy |
| | Thyroid stimulating hormone (TSH) | TRH | |
| | Adrenocorticotropin ACTH | CRH | |
| Female reproductive | | | |

| | | | |
|---|---|---|---|
| Ovaries | Estrogen (E2) and Progesterone (Pr) | FSH, GTH, LH | Gonadogenesis/steroidogenesis menses etc. sexual regulation, pregnancy & menses regulation, embryo development, birth, and lactation |
| Uterus | - | Pr, CG, Lactoferrin (LF) | |
| Mammary | - | Prolactin | |
| Male reproductive | | | |
| Testis<br>Prostate | Testosterone (T) and Dihydrotestrosterone (DHT, 5α-DHT) | - | Spermatogenesis sex hormone functions |
| Thyroid | Thyroxin (T4), triiodothyronine (T3) (free/total), Vitamin A (retinol), linked by transthyretin | TSH | Metabolism, sexual functions, goiter/hypothyroidosis, and epithelial proliferation |
| Adrenal gland (interrenal in invertebrates) | Adrenalin, glucocorticoid Corticosterone (CORT)<br>Cortisol (fish) | ACTH | Energy metabolism at stress combines adrenocorticoid more mineralocorticoid functions |
| Pineal | Noradrenaline (NA), melatonin, serotonin | | (Photo)biorhythms and metabolism |
| Langerhans islets | Insulin | | Glucose metabolism |
| Other systems | Growth H (GH)<br>Antidiuretic H (ADH)<br>(Hepatocyte enzymes, e.g., MFO*, AHH**) | | Growth urophysis<br>(xenobiotic metabolism) |

*MFO=mixed-function oxygenase, **AHH=aryl hydrocarbon hydroxylase.

There are several ways in which Endocrine Disrupting Compounds (EDCs) can act upon an organism, including: mimicking the natural hormone, fooling the body to generate excessive response to stimulation, cause the body to respond at inappropriate times, block the effect of a hormone, directly stimulate or inhibit the endocrine system, and cause un-production or overproduction of the hormone. These possible ways of EDCs acting in the endocrine system are shows in Figure 1.2.

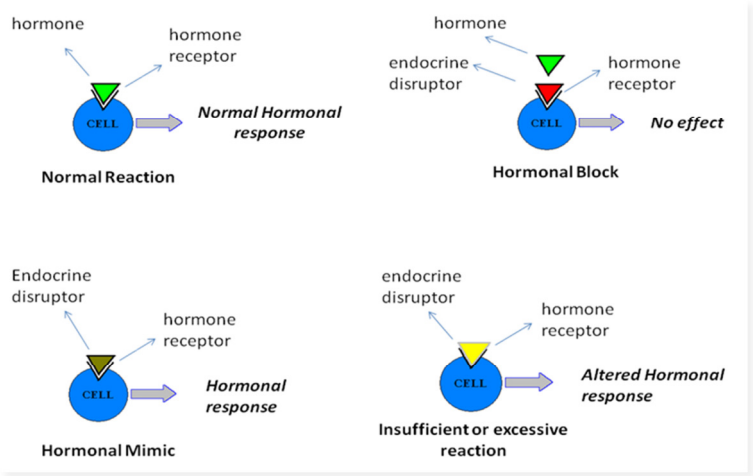



**Figure 1.2 Functional or receptor-based toxicology. Adapted from LaFleur et al. and McLachlan et al. [6,7].**

Generally organisms are subject to EDCs co-exposure.

The results in more or less an additive effect, such as potentiation, synergism, or antagonism, exposure to multiple pesticides may cause changes in the toxicokinetics of the individual compounds, thus modifying the predicted toxicity. Toxicokinetic interactions are the result of one compound altering the absorption, distribution, metabolism or elimination of others and can occur at all dose levels. However, the effects may not be measurable at low doses. The most likely effect of these interactions is the chance in the relationship between the external dose and the corresponding level of pesticide at its target site, leading to an alteration in the threshold [8].

EDCs can contribute to a wide range of diseases and disabilities, including obesity, diabetes, testicular cancer, heart disease, and reproductive health problems (genital malformations, infertility, neuro-development and neurodegenerative disorders). In wildlife EDCs can cause fish and frog feminization, malformations in birds and lizards, among other effects [2,9].

EDCs contain a wide variety of compounds that can come from domestic, industrial, and agricultural pollution. The EDCs are highly heterogeneous and include synthetic chemicals used as industrial solvents/lubricants and their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], and pharmaceutical agents [diethylstilbestrol (DES)]. Natural chemicals found (e.g., phytoestrogens, including genistein and coumestrol) can also act as endocrine disruptors [3,10,11]. The scheme presented in Figure 1.3 shows the different sources of pollution.


**Figure 1.3 Different sources of pollution by endocrine disruptors.**

## 1.1.1 Important factors in endocrine disruption

A number of factors have proven to be key to a full understanding of mechanisms of action and consequences of exposure to EDCs. Several of them are listed below [3].

### 1.1.1.1 - Age at exposure

Exposure of an adult to EDCs may have very different consequences from exposure to a developing fetus or infant. Figure 1.4 shows the different route of exposition to EDCs. In fact, the field of endocrine disruption has embraced the terminology “the fetal basis of adult disease.” This terminology is used to describe observations on how the environment of a developing organism (which includes the maternal environment of eutherian mammals, the eggs of other vertebrates, and the. external environment) interacts with the individual’s genes to determine the propensity of that individual to develop a disease or dysfunction later in life [3].

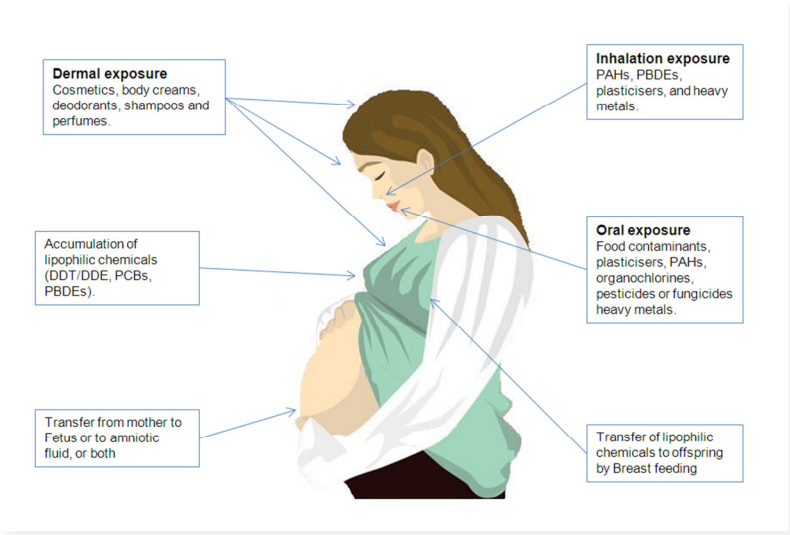



**Figure 1.4 Route of exposition (dermal, inhalation, oral) of the endocrine disruptors. Adapted from Sharpe et al. and Irvine et al. [2].**

### 1.1.1.2 - Latency from exposure

The developmental basis of adult disease also has implicit in its name the concept that there is a lag between the time of exposure and the manifestation of a disorder. In other words, consequences of developmental exposure may not be immediately apparent early in life but may be manifested in adulthood or during aging [3].

### 1.1.1.3 - Importance of mixtures

If individuals and populations are exposed to an EDCs, it is likely that other environmental pollutants are involved as contamination of environment is rarely due to a single compound. Furthermore, effects of different classes of EDCs may be additive or even synergistic [3].

### 1.1.1.4 - Nontraditional dose-response dynamics

There are several properties of EDCs that have caused controversy. Any level of exposure may cause endocrine or reproductive abnormalities, particularly if exposure occurs during a critical developmental window. Surprisingly, low doses may even exert more potent effects than higher doses. EDCs may also exert non-traditional dose-response curves, such as inverted-U or U-shaped curves [3].

### 1.1.1.5 - Transgenerational, epigenetic effects

EDCs may affect not only the individual who is exposed but also their children and succeeding generations. Recent evidence suggests that the mechanism of

transmission may in some cases involve the germline and may be nongenomic. Effects may be transmitted not due to mutation of the DNA sequence, but rather through modifications to factors that regulate gene expression such as DNA methylation and histone acetylation [3].

### 1.1.2 Police of EDCs in Europe

The European Union (EU) is focused on the following: the detection of EDCs in food and environmental samples the impact of these compounds on reproductive health related to the endocrine system, improving assessment methods and tools, and risk management. In this context the EU adopted in December 1999 a strategy to address the problems of EDCs, which produced a list of possible substances with endocrine disrupting activity [4,12].

Priority was placed on any evidence of disruptors in humans and animals, and their potential exposure to the chemical. This was done using the basis of its persistence in the environment, and the amount of substance produced. The disruptors were then grouped according to these criteria.

#### 1.1.2.1 Priority list

The priority list (564 chemicals) was established in two phases. The first phase contains an independent review of evidence of endocrine disrupting effects and human/wildlife exposure.

The second phase contains a priority-setting exercise that involves consultations with stakeholders and Commission Scientific Committees. The process includes different steps and is described in the text below in Figure 1.5. In step 1, a working list of chemicals was compiled from lists of 'suspected endocrine disruptors' that were published by various organisations. This list was then supplemented by a search of the scientific literature to identify reports describing effects that suggested the existence of endocrine disrupting activity in specific chemicals. To try to ensure that the list was as comprehensive as possible, a draft of the list was discussed at a meeting with key stakeholders (including representatives from government, industry and non-governmental organisations (NGOs)).

Data on the effects of chemicals in humans and animals possibly due to endocrine disruption was collected and included in a database to facilitate the analysis of findings. Information on each chemical's persistence in the environment and the likelihood that its levels might build up in exposed organisms (i.e. bioaccumulate) was also collected and made available.

In step 2, the available information was reviewed to identify those chemicals that might be either highly persistent in the environment or are produced by industry as High Product ion Volume (HPV) chemicals, (i.e. more than 1000 tonnes each year). At any rate, it was assumed that both humans and animals would be more likely to be exposed to them and, as a result, be at potentially greater risk to any harmful effects.

In step 3, using expert advice, information on the subset of chemicals identified by step 2 (persistent or HPV chemicals) was reviewed to determine the strength of evidence for endocrine disruption. The chemicals were then assigned to one of three categories. In this priority-setting, Commission Scientific Committees and Stakeholders were consulted and a differentiation between both categories is worth noting. : Category 1 - evidence of endocrine disrupting activity in at least one species using intact animals; Category 2 - at least some in vitro evidence of biological activity related to endocrine disruption; Category 3 - no evidence of endocrine disrupting activity or no data available.

In step 4, regarding the chemicals assigned to Category 1 in Step 3 (i.e. those for which there was evidence of endocrine disrupting activity in at least one intact animal species), the available information was reviewed to decide, if it was possible, that humans or wildlife might actually be exposed.

The highest concern was allotted to examples in which humans and/or wildlife were expected to be exposed, medium concern related to examples in which humans were not expected to be exposed but wildlife could be, and lowest concern was given for those where neither humans or wildlife were exposed.

The European Commission (EC) has compiled a list containing 564 substances, all of which are categorized in Group I, II or III depending on scientific analysis [13]. Table 1.2 shows these Groups of chemicals.

Of the 564 chemicals suggested as being possible EDCs (by several reputable organizations, published scientific articles/reports), 147 were considered likely to be either persistent in the environment or produced at high volumes (assigned Category 1 using the criteria adopted in the study), and 52 chemicals showed some evidence suggesting potential activity (Category 2).

**Table 1.2 List candidate substances-summary of work to date. Adapted from Ch. Groshart et al. [13]**

| Group | Selection criteria | | | Number of substances |
|---|---|---|---|---|
| **Group I** | Highly persistent and/or HPV | Category 1 | High concern in term of human and wildlife exposure | 60 (29 chemicals groups) |
| **Group II** | Highly persistent and/or HPV | Category 1 | Medium concern in term of human and wildlife exposure | 4 |
| | | Category 2 | | 51 |
| **Group III** | Highly persistent and/or HPV | Category 1 | Low concern in term of human and wildlife exposure | 2 |
| | | Category 3 | | 18* |
| | Not HPV and not highly persistent | | | 213 |
| | Not HPV and no data on persistence | | | 205** |

* Excluding 11 Substances that have been excluded from the candidate list because of data giving no basis for inclusion in the list (Category 3)

** No Smiles notations were readily available for QSAR estimations on persistence.

In total, 118 substances were categorised in the first exercise as priority examples. Of the 66 chemicals in Category 1, humans were considered likely to be exposed to 60 of them.

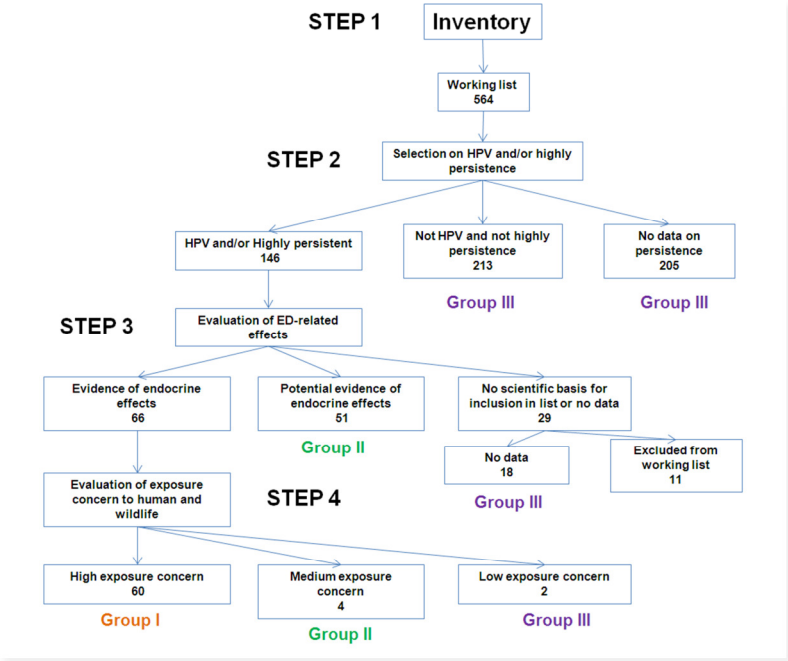



**Figure 1.5 Overview of the steps of the project made by the European commission. Adapted from Groshart et al. [13].**

The majority of substances registered in the list of chemicals adopted by the EC in 2007 are pesticides.

EDCs are considered highly persistent based on related Quantitative Structure–Activity Relationship (QSAR) analysis. This analysis is derived from the combination of two models of biodegradation.

When applying the ultimate degradation model, they are considered highly persistent to those with a low probability of degradation (P <0.1). This model was divided into three subgroups: highly persistent substances (pers +), persistent (pers); non-persistent (not pers).

An important criterion in risk assessment is the bioaccumulation of chemicals by aquatic organisms (especially Daphnia, mussels and fish).

To protect freshwater and marine organisms in their environment, bio-concentration must be considered in context with toxicity, biotic and abiotic degradation, as well as other physical-chemical factors. Furthermore, it is necessary to prevent human exposure from contaminated aquatic food, such as fish, mussels, and oysters [14]. Figure 1.6 shows the possible bioaccumulation of EDCs by aquatic organisms.

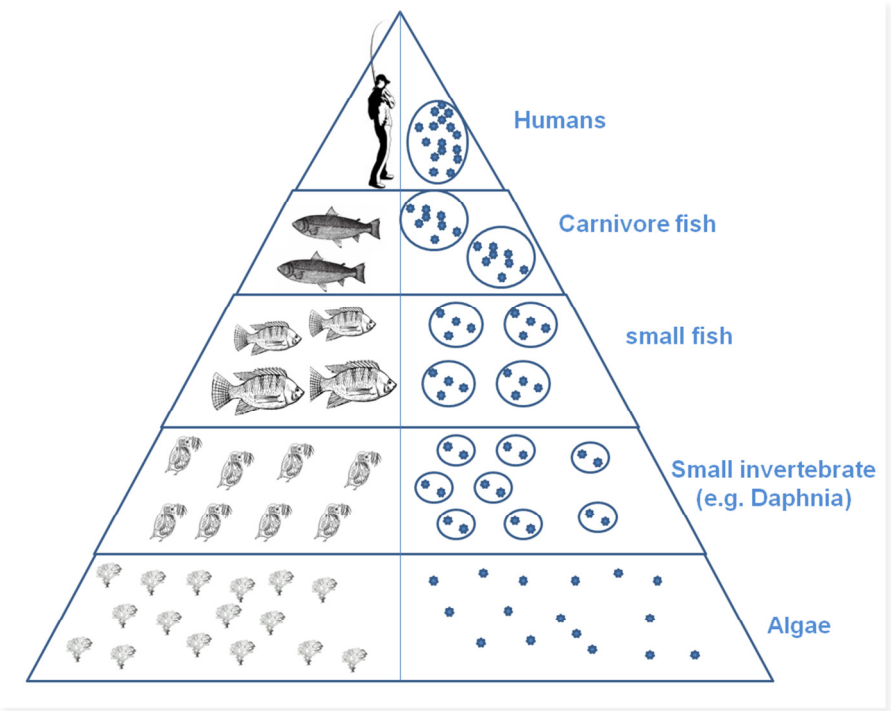



**Figure 1.6 The possible bioaccumulation of EDCs by aquatic organisms.**

## 1.1.3 A particular group of endocrine disruptors - Pesticides

A pesticide is defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest [15].

Recently, the EU classified endocrine disruption properties as a hazard, therefore any plant protection product containing said properties would be considered for an immediate ban.

However, substances showing endocrine disruption properties can be subject to derogation and be approved for maximum of 5 years. This is true only if it can be proven that the substance can be used safely and is necessary to protect plant health [16]. Endocrine disrupting pesticides (EDPs) are the largest group of EDCs numerically in comparison to other chemical groups[17]. Pesticides which reach the soil or crops in target areas may disappear by degradation or dispersion. Pesticides may also volatilise into the air, runoff or leach into surface water and groundwater, be taken up by plants or soil organisms or simply stay in the soil. Figure 1.7 shows possible ways that pesticides are distributed in the environment.

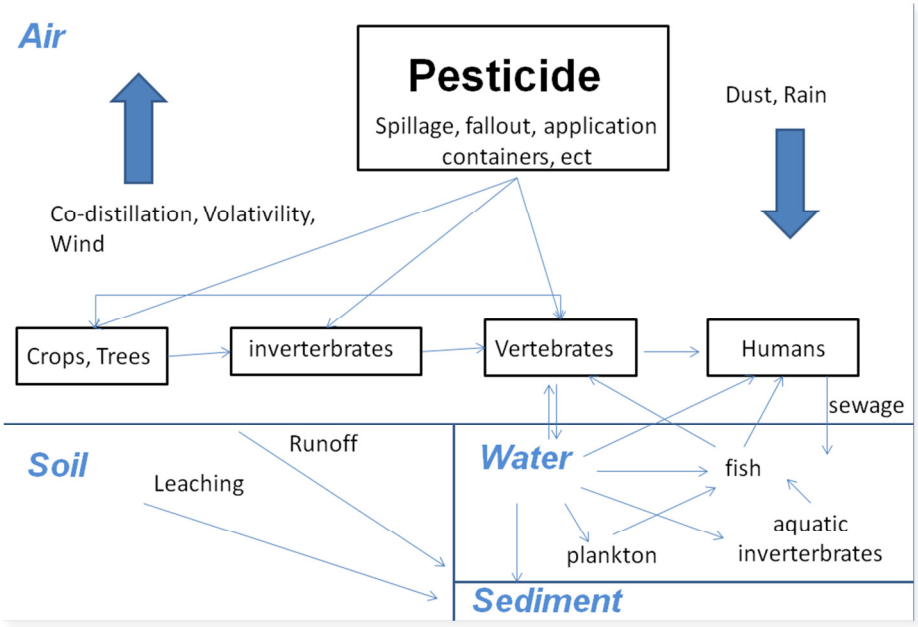


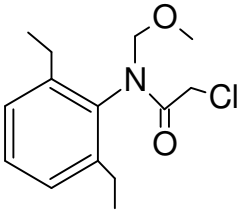
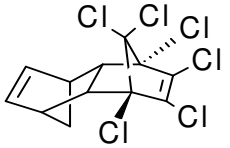
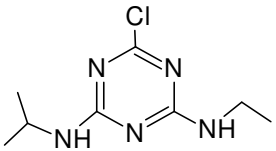

**Figure 1.7 Distribution of pesticides in the environment after it is applied and the various processes involved. Adapted from Zhang et al.[18].**

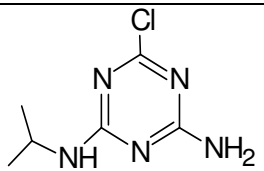
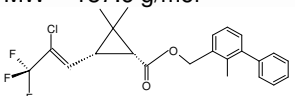
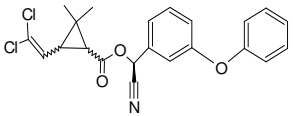
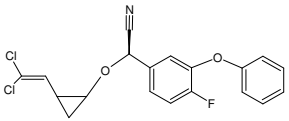
Endocrine disrupting pesticides studied can be found among the families of pyrethroids, organophosphates, imidazoles, triazines and organochlorine. Table 1.3 shows the pesticides studied in this work in which physicochemical properties, chemical structure, and family are categorized according to their possible endocrine disruptor effects, their class and current use are also described.

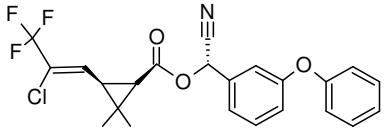
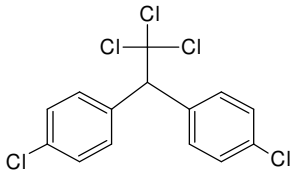
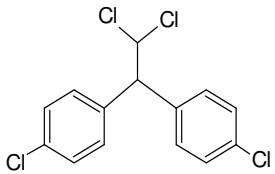
Environmental processes are tremendously complex. The sites of most interest are agricultural fields, forests, lakes, and streams. There are also subtle living ecosystems that are completely, understood and subject to great variability in space and time. The

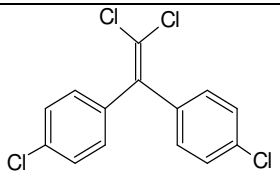
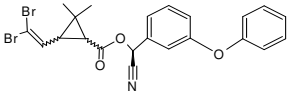
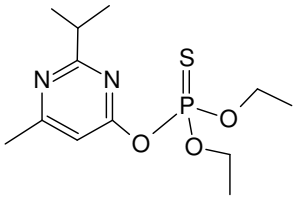
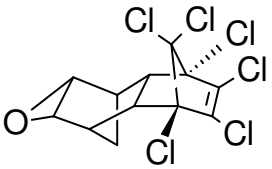
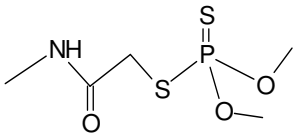
only way to develop a prediction capability is to develop an understanding of the most basic processes driving pesticide dissipation and degradation between environmental conditions. A current approach to predicting water pollution potential is to estimate each chemical´s inherent tendency to undergo leaching or runoff on the basis of its physical and chemical properties [19].

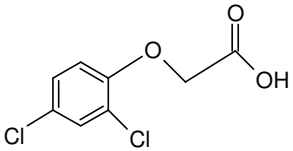
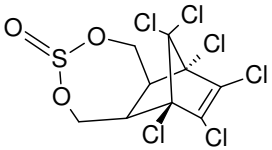
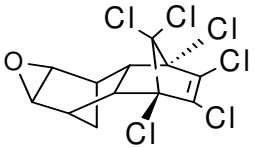
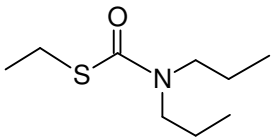
**Table 1.3 Effects of endocrine disruptor pesticides and their chemical structure.**

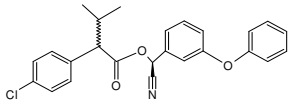
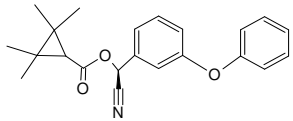
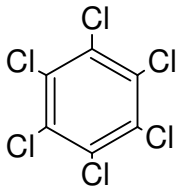
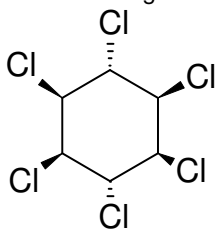
| Pesticides and metabolites | Chemical structure | Endocrine Disruptor Effects | Class and Current use | Ref. |
|---|---|---|---|---|
| **alachlor**<br>pKa = 0.62<br>$pK_{OW}$ = 3.09<br>Vapor Pressure =0.002 Pa (25ºC)<br>Solubility (water) = 170.31 mg/L (pH 7, 20 ºC)<br>Persist = Not pers |  <br>MW = 269.8 g/mol | Synergistic androgenic effects when combined with testosterone<br>In-vitro studies: competitive a ER (estrogenic receptor) and aPR (alligator Progesterone receptor) binding<br>Animal studies: no reproductive effects in adult rats<br>Group: I (High, exposure concern) | Chloroacetamide<br>Herbicide | 1,20-23 |
| **aldrin**<br>pKa = n.a<br>$pK_{ow}$=5.319 (calculated)<br>Solubility (water) = 0.027 mg/L (27ºC)<br>Vapor Pressure = 0.0086 Pa (20ºC)<br>Persist = Pers+ |  <br>MW= 364.9 g/mol | Competitive binding to androgen receptors Antagonises the action of androgens by binding competitively to their receptors and inhibiting the genetic transcription they induce.<br>Group: II | Cyclodiene, organochlorine<br>Non-systemic insecticide | 1,20,21,23 |
| **atrazine**<br>pKa =1.6, 4.14, 10.7<br>logP = –0.97<br>$pK_{ow}$ = 2.5 (25ºC)<br>Vapor Pressure= 3.9 x $10^{-05}$ Pa (25º C)<br>Solubility (water) = 33 mg/L (22ºC, pH 7)<br>Persist = Pers |  <br>MW = 215.7 g/mol | Androgen inhibition, weak estrogenic effect. Disruption of the hypothalamic control. Adrenal glands damages and reduction of steroid hormone metabolism<br>In-vitro studies: weak estrogen, weak anti-androgen,<br>Animal studies: damage to adrenal glands, Impairment of steroid, hormone metabolism<br>Group: I (High, exposure concern) | Triazine<br>Herbicide | 1,20-23 |

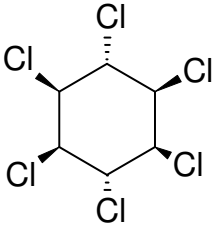
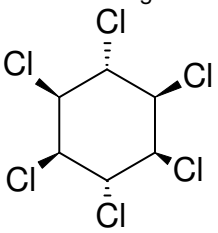
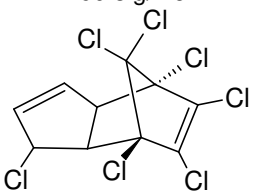
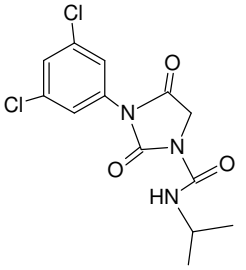
| | | | | |
|---|---|---|---|---|
| **atrazine desethyl**<br>pKa =1.3, 1.65<br>$pK_{ow}$ = 1,504 (calculated)<br>Solubility (water) = 3200 mg/L (22ºC)<br>Persist =- |  <br>MW = 187.6 g/mol | | Triazine<br>metabolite of atrazine, cyprazine, and propazine) | 21 |
| **bifenthrin**<br>pKa = Non ionised<br>$pK_{ow}$ = 6.6 (20 ºC, pH 5)<br>Vapor pressure = $1.78 \times 10^{-05}$ Pa (20 ºC)<br>Henry's constant = $7.2 \times 10^{-3}$ atm·$m^3$ /mol<br>Solubility (water) = 0.001 mg/L (20ºC)<br>Soil Sorption Coefficient (Koc) = $1.31 \times 10^5$ – $3.02 \times 10^5$<br>Persist = Pers |  <br>MW = 422.9 g/mol | Interferes with the action of the female sex hormones, causing reductions in ovary weight and lack of oestrus. Decreases the level of thyroid hormones present in the blood.<br>Group: III | Pyrethroid<br>Insecticide, acaricide | 1,21,23,24 |
| **cypermethrin**<br>pKa = Non-ionised<br>$pK_{ow}$ = 5.3-5.6 (25ºC)<br>Vapour pressure = $2.3 \times 10^{-07}$ Pa (20 ºC)<br>Solubility (water) = 0.009 mg/L<br>Persist = Not pers |  <br>MW = 416.3 g/mol | Estrogenic effect<br>Androgen receptor may be involved in the adverse effects of β-cypermethrin on the reproductive system of male rats.<br>Mimics the action of oestrogen. Metabolites also have oestrogenic effects.<br>Group: III | Pyrethroid<br>Insecticide | 1,20,21,23,25 |
| **β-cyfluthrin**<br>pKa = Non-ionised<br>$pK_{ow}$=5.9 (22ºC)<br>Solubility (water) = 0.0012 mg/L (20ºC) |  <br>MW= 434.3 g/mol | Antiandrogenic effects in vitro and in vivo. | Pyrethroid<br>Insecticide | 21,26 |

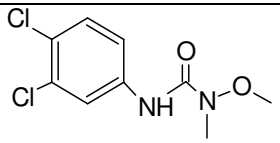
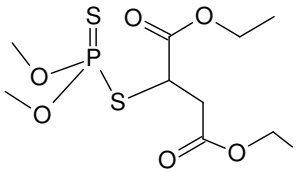
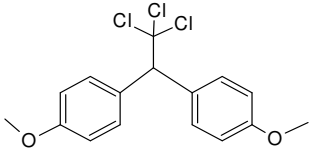
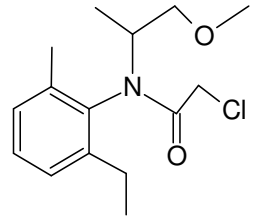
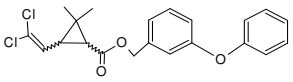
| | | | | |
|---|---|---|---|---|
| Vapor pressure = $8.5x10^{-08}$ Pa (20ºC)<br>Persist = - | | | | |
| **λ-cyhalothrin**<br>pKa = Non-ionised<br>Solubility (water) =0.005 mg/L (20ºC, pH 6.5)<br>$pK_{ow}$= 7 (20 ºC)<br>Vapour pressure = $2x10^{-07}$ Pa (20ºC)<br>Persist = Not pers |  <br>MW = 449.9 g/mol | Decreases the secretion of thyroid hormones.<br>Group: III | Pyrethroid<br>Insecticide, acaricide | 1,21 |
| **DDT**<br>pKa = n.a<br>$pK_{ow}$ = 6.91<br>Solubility (water) = Virt. Not soluble<br>Vapour pressure = $2.5x10^{-05}$ Pa (25ºC)<br>Persist= Pers |  <br>MW = 354.5 g/mol | Competitive binding to androgen receptors, activation of androgen-sensitive cells proliferation.<br>In-vitro studies: estrogen, anti-androgen<br>Animal studies: estrogenic effects thyroid inhibition<br>Stimulation of estrogen receptor production, estrogen receptor agonist and PR antagonist<br>Group: I (technical), III (chemo) | Organochlorine<br>Insecticide | 1,20-23 |
| **o,p-DDT and p,p-DDT**<br>$pK_{ow}$ = 5.923 (calculated)<br>**o,p-DDD**<br>$pK_{ow}$ = 5.389 (calculated)<br><br>**p,p-DDD**<br>M(g/Mol) = 320.05<br>Solubility (water) = 0.05 mg/L (20 ºC) Not soluble<br>$pK_{ow}$ = 5.389 (calculated)<br>Persist= Pers |  <br>MW = 320.05 g/mol | | Organochlorine<br>metabolite of DDT | |

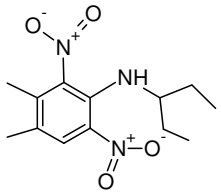
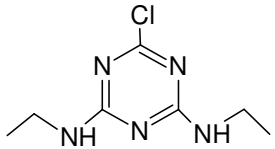
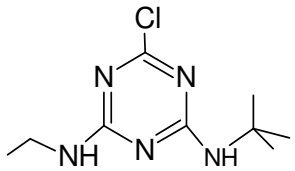
| | | | | |
|---|---|---|---|---|
| **o,p-DDE**<br>$pK_{ow}$ = 6.223<br>(calculated)<br><br>**p,p-DDE**<br>$pK_{ow}$ = 6.369<br>(calculated)<br>Persist= Pers |  <br>MW = 318.03 g/mol | ER is sensitive to DDE | Organochlorine | 27 |
| **deltamethrin**<br>pKa = Non-ionised<br>$pK_{ow}$ = 4.6 (25ºC)<br>Solubility (water) =<br>0.0002 mg/L (25 ºC)<br>Vapor pressure =<br>$1.24x10^{-08}$ Pa (25ºC)<br>Persist = Not pers |  <br>MW = 505.2 g/mol | Weak estrogenic activity.<br>Group: III | Pyrethroid<br>Insecticide | 1,20,21,23 |
| **diazinon**<br>pKa = 2.6<br>$pK_{ow}$ = 3.69 (24ºC)<br>Solubility (water) = 60<br>mg/L (22 ºC)<br>Vapour pressure =<br>0.01197 Pa (25ºC)<br>Persist = Not pers |  <br>MW = 304.4 g/mol | Estrogenic effect<br>Mimics the action of oestrogen.<br>Group: II | Organophosphorous<br>Insecticide, acaricide | 20,21,23 |
| **dieldrin**<br>pKa = n.a<br>$pK_{ow}$ = 4.879<br>(calculated)<br>Solubility (water) =<br>0.186 mg/L (20 ºC)<br>Vapour pressure =<br>0.0004 Pa (20ºC)<br>Persist= Pers+ |  <br>MW = 380.9 g/mol | Competitive binding to androgen receptors, estrogenic effect, stimulation of estrogen receptor production<br>In-vitro studies: weak estrogen, weal anti-androgen<br>Animal studies: no reproductive effects<br>Group: II | Cyclodiene,<br>organochlorine<br>Insecticide and<br>(Metabolite of aldrin) | 1,20-23 |
| **dimethoate**<br>pKa = Non-ionised<br>$pK_{ow}$ = 0.704 (pH 7)<br>Solubility (water) |  | Disruption of thyroid hormones action. Increase of insulin blood concentration, decrease of luteinizing hormone blood concentration<br>Human studies: reduced fecundability<br>Group: II | Organophosphorous<br>Insecticide | 1,20-23 |

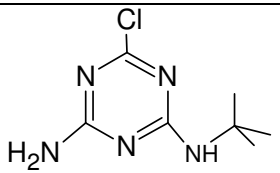
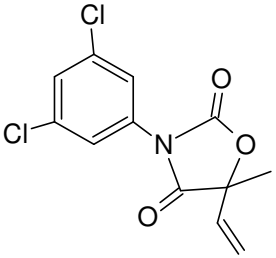
| | | | | |
|---|---|---|---|---|
| =39800 mg/L (25 ºC, pH 7)<br>Vapour pressure = 0.00025 Pa (20ºC)<br>Persist = Not pers | MW = 229.3 g/mol | | | |
| **2,4-D**<br>pKa = 2.73<br>logP = 2.81 |  <br>MW = 221 g/mol | Synergistic androgenic effects when combined with testosterone | Aryloxyalkanoic acid/ester | 21 |
| **(α and β) endosulfan**<br>pKa = n.a<br>$pK_{ow}$ = (α) 4.74 and (β) 4.79<br>Solubility (water) = (α) 0.32 and (β) 0.33 mg/L (22 ºC)<br>Persist = Pers+ |  <br>MW = 406.9 g/mol | Antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce.<br>Mimics the actions of oestrogens in directly by stimulating the production of their receptors. Weak aromatase inhibitor. Competitive binding to androgen receptors<br>In-vitro studies: Weak estrogen. Competitive aER and aPR binding. Impaired steroid synthesis in Leydig cells<br>Animal studies: Damage to seminiferous tubules in male rats and reproductive organs in female mice<br>Group: II | Organochlorine Insecticide, acaricide | 1,20-23 |
| **endrin**<br>pKa= Non-ionised<br>$pK_{ow}$ = 4.879 (calculated)<br>Solubility (water) = Virtually not soluble<br>Vapour pressure = $2x10^{-08}$ Pa (20ºC)<br>Persist= Pers+ |  <br>MW = 380.9 g/mol | Antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce<br>Group: II | Organochlorine Foliar insecticide | 1,20,21,23 |
| **EPTC**<br>pKa= n.a<br>$pK_{ow}$ = 3.2<br>Solubility (water) = 375 mg/L<br>Vapour pressure = 10 Pa (25ºC) |  <br>MW = 189.3 g/mol | EDCs disrupt ER-α signaling | Thiocarbamate Herbicide | *21,28* |

| | | | | |
|---|---|---|---|---|
| Persist = - | | | | |
| **fenvalerate**<br>pKa = n.a<br>$pK_{ow}$ = 5.01<br>Persist = Not pers |  <br>MW = 419.9 g/mol | Inhibition of estrogen-sensitive cells proliferation, antagonist of the progesterone action.<br>Inhibits the proliferation of oestrogen-sensitive cells, antagonizes the action of progesterone.<br>Group: III | Pyrethroid<br>Insecticide, acaricide | 1,20,21,23 |
| **fenpropathrin**<br>pKa= Non-ionised<br>$pK_{ow}$ = 6 (20ºC)<br>Solubility (water)= 0.014 mg/L (25ºC)<br>Vapour pressure = 0.00073 Pa (20ºC)<br>Persist:- |  <br>MW= 349.4 g/mol | In vitro: Weak estrogenic and antiandrogenic activity observed | Pyrethroid<br>Insecticide, acaricide | 21,29 |
| **HCB**<br>pKa = n.a<br>pKow= 5.66<br>Vapour pressure= 0.00145 Pa (20ºC)<br>Persist= Pers |  <br>MW = 284.8 g/mol | Severely disruption of thyroid hormone production. Enhancement of androgen action at low doses, but inhibition at high ones.<br>Animal studies: reduced fertility<br>Human studies: Changes in the levels of sex hormones.<br>Group: I | Organochlorine<br>Fungicide, insecticide | 1,20-23 |
| **HCH (lindane)**<br>pKa= Non-ionised<br>$pK_{ow}$ = 3.5<br>Solubility (water) = 8.52 mg/L (25ºC)<br>Vapour pressure = 0.0044 Pa (24ºC)<br>Persist = Pers |  <br>MW = 290.8 g/mol | Severely disrupts thyroid hormone production. Enhances androgen action at low doses, but inhibits it at high ones. Reduction of oestrous cycles and luteal progesterone concentrations. Increase of insulin and estradiol concentrations, decrease thyroxine concentrations.<br>Competitive binding to AR(androgen receptor), ER and PR<br>In-vitro studies: weak anti-androgen, no estrogen effect, impired steroid synthesis in Leydig cells<br>Animal studies: Testitular atrophy decreased sperm production and testosterone levels in rats and mink.<br>Group: I | Organochlorine<br>Insecticide | 1,20-23 |

| | | | | |
|---|---|---|---|---|
| **α-HCH**<br>pKa= Non-ionised<br>$pK_{ow}$ = 3.99 (calculated)<br>Persist =Pers<br>Insecticide |  <br>MW = 290.8 g/mol | In-vitro studies: Weak anti-androgen | Organochlorine<br>Insecticide | 21,22 |
| **β-HCH**<br>pKa= Non-ionised<br>$pK_{ow}$ = 3.99 (calculated)<br>Persist = Pers |  <br>MW= 290.8 g/mol | Animal studies: Weak estrogen-like effects in mice and rats.<br>Group: III | Organochlorine<br>Insecticide | 1,21,22 |
| **heptachlor**<br>pKa = Non-ionised<br>$pK_{ow}$ =4.4-5.5<br>Solubility (water)= 0.056 mg/L (25-29ºC)<br>Vapour pressure = 0.053 Pa (25ºC)<br>Persist = Pers+ |  <br>MW= 373.3 g/mol | Binding to cellular estrogen and androgen receptors.<br>Group: II | Cyclodiene, organochlorine<br>Non-systemic insecticide | 1,20,21,23 |
| **iprodione**<br>pKa = Non-ionised<br>$pK_{ow}$ =3 (pH 3, pH 5)<br>Solubility (water) = 12.2 mg/L (20ºC, pH 7)<br>Vapour pressure = 5 x $10^{-07}$ Pa (25ºC)<br>Persist = Not pers |  <br>MW = 330.2 g/mol | Increase weakly aromatase activity, and estrogen production<br>Group: II | Dicarboximide<br>Fungicide | 1,20,21,23 |

| | | | | |
|---|---|---|---|---|
| **linuron**<br>pKa = Non-ionised<br>$pK_{ow}$ = 3 Solubility (water) = 63.8 mg/L (20ºC, pH 7)<br>Vapour pressure = 0.0051 Pa (25ºC)<br>Persist = Not pers |  <br>MW = 249.1 g/mol | Competitively inhibits the binding of androgen to its receptor, inhibits androgen-inducing gene expression. Alters androgen-dependant ventral prostate gene expression.<br>Group: I (High, exposure concern) | Urea<br>Herbicide | 1,20,21,23 |
| **malathion**<br>pKa = Non-ionised<br>$pK_{ow}$ = 2.75 Solubility (water) = 148 mg/L (25ºC)<br>Vapour pressure = 0.00045 Pa (25ºC)<br>Persist = Not pers |  <br>MW = 330.4 g/mol | Inhibition of catecholamine secretion, binding to thyroid hormone receptors.<br>Group: II | Organophosphorous<br>Insecticide, acaricide | 1,20,21,23 |
| **methoxychlor**<br>pKa = n.a<br>$pK_{ow}$ =4.83 Solubility (water) = 0.1 mg/L (25ºC)<br>Persist = Not pers |  <br>MW = 345.7 g/mol | Strong estrogenic effect. Also antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce. Interacts with the pregnane X cellular receptor, interfering with the manufacture of enzymes responsible for steroid hormone metabolism.<br>Effects animal: Testicular atrophy in rats, no reproductive effects in mice.<br>Group: III | Organochlorine<br>Insecticide y herbicide. | 1,20-23 |
| **metolachlor**<br>pKa = Non-ionised<br>$pK_{ow}$ = 2.9 (25ºC)<br>Solubility (water) = 490 mg/L (25ºC)<br>Vapour pressure = 0.0042 Pa (25ºC)<br>Persist =- |  <br>MW= 283.8 g/mol | Pregnane X cellular receptor activation, these findings suggest that some EDs affect sex hormone receptor indirectly by induction of metabolic enzyme via PXR, to produce rapidly higher concentrations of effective metabolites, leading to disturbance of the endocrine system. | Chloroacetamide<br>Herbicide | 20,21,30 |
| **permethrin (cis and trans)**<br>$pK_{ow}$ = 6.1 (20ºC)<br>Solubility (water) = 0.006 mg/L (20ºC, pH |  <br>MW = 391.3 g/mol | Inhibition of estrogen-sensitive cells proliferation<br>Metabolites also have oestrogenic effects.<br>Group: III | Pyrethroid<br>Insecticide | 1,20,21,23 |

| | | | | |
|---|---|---|---|---|
| 7)<br>Persist = Not pers | | | | |
| **pendimethalin**<br>pKa = 2.8<br>$pK_{ow}$ = 5.2 (pH 7)<br>Solubility (water) =0.33 mg/L (20ºC)<br>Vapour pressure = 0.00194 Pa (25ºC)<br>Persist = Pers |  <br>MW = 281.3 g/mol | Pendimethalin is known to affect the pituitary-thyroid-axis in humans which means that this substance may be a potential endocrine disruptor. Although slight fluctuations in thyroid hormone levels have been noted in rats, chronic toxicity studies in three different animal species demonstrated no apparent oestrogenic effects or treatment related effects on any other component of the endocrine system. Bohmler and Borowski (2004) tested pendimethalin for oestrogenic activity using the Escreen which examines the proliferative effect of oestrogens on their target cells as the endpoint. This assay uses MCF-7 cells and compares the cell number achieved in the absence of oestrogens (negative control), in the presence of 17β-oestradiol (positive control) and in the presence of the substance under investigation. Pendimethalin was found to be a partial oestrogen receptor agonist. Further studies may be required to determine whether pendimethalin has endocrine-disrupting effects in vivo at environmental concentrations.<br>Group: III | Dinitroaniline Herbicide | 1,21,31 |
| **simazine**<br>pKa = 1.62<br>$pK_{ow}$ = 2.1 (25ºC)<br>Solubility (water) = 6.2 mg/L (20ºC, pH 7)<br>Vapour pressure = 2.94 x $10^{-06}$ Pa (25ºC)<br>Persist = Not pers |  <br>MW = 201.7 g/mol | Induction of aromatase activity, increase of estrogen production.<br><br>Animal studies: no reproductive effects in rats. Distrophy and necrosis of germ cells in sheep.<br>Group: II | Triazine Herbicide | 1,20-23 |
| **Terbuthylazine**<br>pKa = 2<br>$pK_{ow}$ = 3.4 (25ºC)<br>Solubility (water) = 9 mg/L (20ºC, pH 7.4)<br>Vapour pressure= 9 x $10^{-05}$ Pa (25ºC)<br>Persist = - |  <br>MW = 229.7 g/mol | In vitro, TBA shows weak to moderate human PXR activation; as a nuclear receptor, PXR acts as a transcription factor and, following ligand binding, functions as a heterodimer with retinoid X-receptor (RXR) in a nonpermissive way, and is thus involved in the metabolism of endogenous and exogenous compounds. | Triazine Herbicide | 21,32 |

| | | | | |
|---|---|---|---|---|
| **terbuthylazine-desethyl**<br>$pK_{ow}$ = 1.853<br>Persist = - |  <br>MW= 201.7 g/mol | | Triazine<br>Metabolite of terbutilazine | 21 |
| **vinclozolin**<br>pKa = Non-ionised<br>$pK_{ow}$ = 3.4 (pH 7)<br>Solubility (water) = 2.6 mg/L (20ºC)<br>Vapour pressure = 0.00013 Pa (20ºC)<br>Persist = pers |  <br>MW = 286.1 g/mol | Potent androgen-receptor antagonist. Competitively inhibits the binding of androgen to its receptor and inhibits androgen-inducing gene expression. Interfere with the synthesis of enzymes responsible for steroid hormone metabolism.<br>Animal studies: desmaculinizing effects in rats, no reproductive effects in rats.<br>Group: I (High, exposure concern) | Dicarboxymide<br>Fungicide | 1,20-23 |

## 1.1.4 Portugal and endocrine disrupting pesticides (EDPs)

In Portugal, the agency in charge of determining which pesticides are approved for use is the Direção-Geral de Alimentação e Veterinária do Ministério da Agricultura, Mar, Ambiente e Ordenamento do Território, abbreviated as DGADR, fixed by July 31 of each year to control pesticides by managers in the following year[33].

The ERSAR website gives a list of pesticides that should be analysed in each region based on the crops, pests and the advice given to farmers. In Portugal, the legislation applied to 33 priority substances and the other pollutants defined by the EC for surface waters is regulated by the Decree law (DL) 103/2010. This is in line with European Union Directive 2008/105/EC. For drinking water the DL 306/2007 is related to the International Directive 98/83/EC [34-39].

Pesticide exposure in Portugal farming areas was reported in 2009 by the Instituto Nacional de Estadistica (INE) [40]. According to the 2009 report, conventional farming areas in northwest Portugal were the main focus of the study. Figure 1.8 shows the risk to the environment posed by drivers of agriculture in Portugal as published by INE [40].

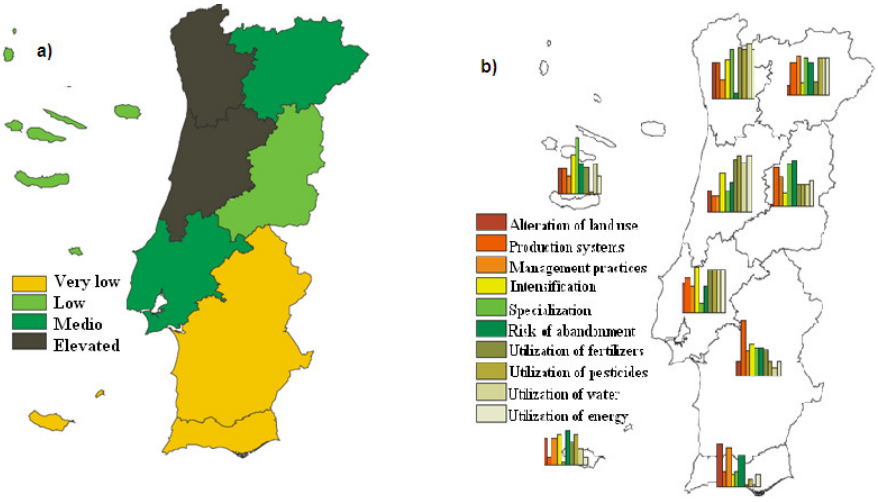



**Figure 1.8 a) Relative risk to the environment posed by drivers of agriculture, and b) Importance of each indicator "driving forces" the assignment of the relative risk to the environment, by Region in Portugal. Adapted from reported INE 2009 [40].**

The studies reported in

Table 1.4 shows that the Portuguese population are exposed to pesticides. However, it is not known how pesticides can affect the population. Most of the detected pesticides are below the limits established by the EU. Nevertheless the combination of pesticides is a subject to consider further and is of utmost importance to monitor these compounds in the environment.

**Table 1.4 Pesticides background detected in different types of samples is Portugal.**

| Place | Samples | Pesticides detected | Ref. |
|---|---|---|---|
| Sado Estuary | Tissues of the oyster (*Crassostrea angulata)* | DDT-total | 41 |
| Alvalade do Sado, Monte da Vinha, Monte Real, Povoas e Meades, Ponte Aranha, Ponte Carvoeira, and Cais do Alcoutim. | Water river | Atrazine, simazine, terbuthylazine, alachlor, metolachlor, Irgarol, propanil; tributhylphosphate, diuron, 2,4,6-trichlorophenol, deisopropylatrazine, and deethylatrazine | 39 |
| River Tejo, river Sado. | Surface water (river basins) | Atrazine, E- and Z- isomers of chlorfenvinphos, α-and β-endosulfan, lindane, molinate, and simazine | 36 |
| Ribatejo e Oeste | Ground water (7 wells) | Alachlor, atrazine, metolachlor, and simazine métribuzine | |
| Beira Litoral and Ribatejo e Oeste | Ground water | Atrazine, desethylatrazine, desisopropylatrazine, simazine, alachlo, metolachlor, metribuzin, 3,4-dichloroaniline, dimethoate, α and β-endosulfan, lindane, molinate, and prometryn. | 42 |
| Coimbra | Blood | Endosulfan sulfate, p,p'-DDE, o,p'-DDT, and p,p'-DDD | 35 |
| Central zone of Portugal | Honey | HCB, HCH, and DDT-total | 43 |
| Northern region | Grape skin and in the whole grape | Pyrimethanil | 44 |
| Ria de Aveiro | muscle and liver of sea bass (*Dicentrarchus labrax*) | DDT | 45 |
| Póvoa de Varzim | Soil | Lindane, dieldrin, endosulfan, endosulfan sulfate, 4,4′-DDE, 4,4′-DDD, atrazine, desethylatrazine, alachlor, dimethoate, chlorpyrifos, pendimethalin, procymidone and chlorfenvinphos | 46 |
| Atlantic west Portuguese coast | Muscle of three fish species: European pilchard (*Sardina pilchardus*), Atlantic horse mackerel (*Trachurus trachurus*), and Atlantic mackerel (*Scomber scombrus*) | DDT, HCH, and aldrin | 47 |
| Alqueva | aquatic systems | Atrazine, simazine, terbutylazine, metolachlor, chlorpyrifos, and endosulfan sulphate | 48 |
| Between Esposende and Vila do Conde | Ground water | Most frecuently detected were lindane, pendimethalin, endosulfan sulfate, and endosulfan | 49 |

| | | | |
|---|---|---|---|
| Alqueva | Surface water | atrazine, simazine, diuron, and terbuthylazine | [38] |
| Sado, Sagres, Ría Formosa, Guadiana, Sines, Duoro, Lima, Aveiro, Mondego, Tejo, Porto de Carvoeira, Herdade Portancho, Esteiro de Coina, Ponte Minhoteira, Ardilha fronteira, Oeiras (Alcácer Rio Sado), Olhao porto de pesca, S. Romao do Sado, Porto Carvoeira, and Albufeira pocino | Rivers sediments | Lindane, fenitrothion, parathion-methyl, diazinon, and simazine | [34] |
| Minho estuary, Lima estuary, Cávado estuary, Ave estuary, Douro estuary, Sado estuary, and Ria Formosa | Coastal sites and sediment | Lindane, α-endosulfan , DDE, dieldrin, DDD, and DDT | [37] |
| Central Portugal | Strawberries (from Organic farming and Integrated pest management) | Lindane and β-endosulfan, aldrin, o,p´-DDT and their metabolites, and methoxychlor | [50] |
| North of Portugal (Fontelas, Loureiro, Cederma, Oliveira, Galfura, Mesão Frio, Vila Marim, Vilariho and Canelas . | Ground water | Folpet, 2,4 D, atrazine desethyl, terbuthyllazin-desethyl, dimethoate, terbuthyllazin, dieldrin, endrin, o.p´-DDT, meyhoxychlor, | [51] |
| Porto, Portugal | Human adipose tissue (from Hospital de São João) | HCB, o,p'-DDT, and methoxychlor | [52] |
| Tejo River basin | Water-sediment | Alachlor, atrazine ethofumesate, metolachlor, terbuthylazine, chlorfenvinphos, chlorpyrifos, and the metabolite 3,4-dichloroaniline | [53] |
| Tejo | Ground water | Atrazine, alachlor, metolachlor, desethylatrazine, ethofumesate, α-endosulfan, metribuzine, lindane, and β-endosulfan | [54] |
| Central Iberian Zone | Soil | Aldrin, α-endosulfan, β-endosulfan, heptachlor epoxide, and heptachlor | [55] |
| Pateira de Fermentelos, Aveiro | Water | DDE and α-HCH | [56] |
| Nazaré, Algarve, Alentejo, Vagos, Trás os montes, Guarda and between Douro- Minho. | Carrots | β-HCH | [57] |

## 1.2 Sample Preparation - Environmental

The importance of green chemistry should be noted as competition to chemical products and processes. It effectively reduces or eliminates the use or generation of hazardous substances. Green chemistry uses a set of guiding principles and methods for reducing pollution. .Sample preparation is the step after samples have been collected and preserved

but before samples are introduced into instrument for further analysis [58]. The purpose(s) of environmental sample preparations can be one or a combination of the following steps; a) homogenize sample or remove moisture, b) increase/decrease analyte concentration, c) remove interfering chemicals, d) change sample phase, e) liberate analyte from sample matrix, and f) modify chemical structure [58].

This chapter describe in more detail the extraction techniques for liquid and solid samples used in the experimental part of this thesis and also a briefly review of others techniques described in literature.

Due to low concentrations levels of pesticides found in liquid samples in the environment (using trace levels in the range of nanograms per liter in water), the development of reliable and efficient analytical methods is required. Liquid-liquid extraction (LLE) was the first approach for extraction and continues to be used. Other extraction techniques were developed, including: Micro Liquid-Liquid Extraction (MLLE), Dispersive Liquid-Liquid Microextraction (DLLME), Solid Phase Extraction (SPE), and Solid Phase Microextraction (SPME).

To evaluate the pesticides in the environment another kind of solid samples include soil, sludge, and sediments. Solid samples are a complex and heterogeneous matrix with a porous structure that contains both inorganic and natural organic components. Currently, soils and sediments are subject to intensive pollution by chemical compounds. It is important to note that soils play a vital role in the environment by preventing the contamination of adjacent ecosystems [59]. Conventional extractions applied to these solid samples are SPE, extraction liquid, ultrasonic, and Soxhlet. Between The newest techniques are also used, including: Accelerated Solvent Extraction (ASE), Microwave Assisted Extraction (MAE), and Supercritical Extraction (SFE).

## 1.2.1 Liquid-Liquid Extraction (LLE)

LLE was one sample preparation technique used in analytical chemistry. Chemists have used LLE techniques for over 150 years for isolating organic substances from aqueous solutions. LLE is based on the partition between the aqueous sample compounds and an immiscible organic solvent. One problem that tends to occur with this method has to do with the formation of an emulsion and the amount of organic solvent used [60]. The main disadvantage of this technique is that there is a large amount of organic solvent used with an undesirable environmental impact. The technique also has limited selectivity, and the cleaning and enrichment of the analyte is necessary [61].

## 1.2.2 Micro Liquid-Liquid Extraction (MLLE)

MLLE involves a simultaneous extraction and concentration of the analytes. In this method the water is at a large volume, and it uses a small amount of organic solvent resulting in an extract which can be injected directly in the chromatographic column without further treatment [60]. MLLE is a simple implementation of LLE, and is used as an extraction method for detecting environmental pollutants.

## 1.2.3 Dispersive Liquid-Liquid Microextraction (DLLME)

DLLME is one of the simplest extraction procedures. However, due to the need for several manual manipulations, the technique cannot be completely automated [62].

If a solvent heavier than water (such as carbon tetrachloride or carbon disulfide) is used, a conical - bottomed vial or centrifuge tube is utilized, and the sample is centrifuged to separate the extraction solvent. The sample is then removed with a syringe and analysed [62].

DLLME is based on a ternary component solvent scheme. A cloudy mixture (microdroplets) is formed when a mixture of an extractant (typical non-miscible organic solvent used in classical LLE) or ionic liquids and disperser solvents (miscible organic solvents e.g. methanol, acetone, acetonitrile etc.) is rapidly injected into an aqueous sample. Due to the large contact surface area of the two immiscible phase's high extraction efficiency is achieved in a relatively short time. After formation of a cloudy solution, infinite surface area between the extraction solvent and aqueous phase will be the result. Subsequently, extraction solvent can be separated by centrifugation.

DLLME has a lot of advantages including low cost, low consumption of organic solvent, and a high enrichment factor. However, it is not suitable for extraction of compounds from solid samples [63,64].

The disadvantages of this extraction lie in the difficulties in automation and in the need to use a third component (dispersion solvent). These issues usually decrease the partition coefficient of the analyte in the extractant [65].

## 1.2.4 Solid-Phase Extraction (SPE)

SPE is based on the different affinity of target anlytes for two different phases. In SPE, a liquid (sample liquid or liquid sample extracts) is loaded onto a solid sorbent (polar, ion exchange, non-polar, affinity). Those compounds with higher affinity for the sorbent will be retained on it, whereas other will pass through it unaltered. Subsequently, if target analytes are retained, they can be eluted using a suitable solvent with a certain degree of selectivity [66].

SPE procedures involve typically four steps as described further, 1) the sorbent needs to be prepared by conditioning by activation with suitable solvent and by conditioning with same solvent in which analytes are dissolved, 2) then the samples that are in solution are loaded onto the cartridge, 3) usually, target analytes are retained together with other components of the sample matrix. Some of these compounds can be removed by application of a washing, 4) analytes are eluted with small volume of an appropriate solvent [66]. In Figure 1.9 different steps of this extraction are shown.

Some of the aspects to be considered in SPE are the mobile phase, stationary phase, and the analyte characteristics, in the case of mobile phase molecules that compete effectively with analyte molecules for the attractive stationary phase sites displace these analytes, causing them to move faster through the column (weakly retained). Water is at the polar end of the mobile-phase-solvent scale, while hexane, an aliphatic hydrocarbon, is at the non-polar end. In between, single solvents, and miscible-solvent mixtures can be placed in order of elution strength. Which end of scale represents the strongest mobile phase depends upon the nature of the stationary phase surface where the completion for the analyte molecules occurs. For example in normal-phase application with a polar sorbent, water is a strong solvent because it is also polar and can release analytes from the sorbent. If it is a reversed-phase application with a non-polar sorbent, then water is weak solvent, since it cannot release non-polar analytes that are attracted to the sorbent [67]. Another important factor is the stationary phase; unbounded silica has an active, hydrophilic (water-loving) polar surface containing acidic silanol (silicon-containing analog of alcohol) functional groups. Consequently, it falls at the polar end of the stationary-phase scale. The activity or polarity of the silica surface may be modified selectively by chemically bonding to it less polar groups (bonded phase) [67]. Finally analyte characteristics are essential to chosen the best combination of a mobile phase and stationary phase. For example some compounds are very polar, such as salts and charged acids, while other analytes, are very non-polar such as flavouring oils [67].

Principal separation modes used in SPE are following normal phase, reversed phase, and ion-exchange [68]. In normal-phase chromatography, the stationary phase is polar and retains the polar dye most strongly. The relatively non-polar dye is more attracted by the mobile phase, a non-polar solvent, and elutes quickly [67]. Reverse-phase describes the chromatography mode that is just the opposite of normal phase, namely the use of a polar mobile phase and a non-polar (hydrophobic) stationary phase. In the case ion-exchange the extraction of charged analytes from aqueous or non-polar organic samples [68].

During the last two decades, SPE has steadily gained acceptance within the analytical community and is now rapidly replacing traditional LLE as the sample preparation techniques of choice. The efficacy and economy (in solvents) of SPE is now well documented in a staggering number of peer-reviewed articles [60].

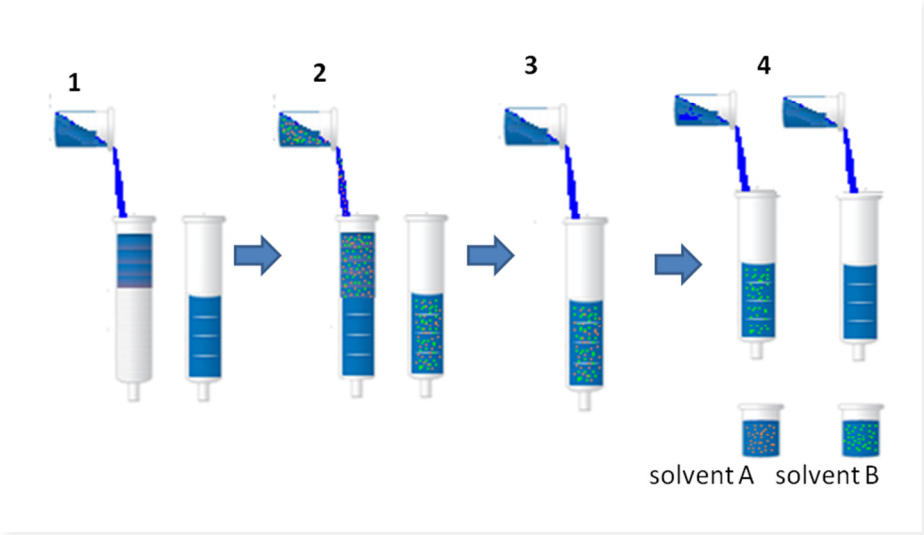



**Figure 1.9 Different steps of extraction for SPE. 1) Cartridge is equilibrated or conditioned with a solvent to wet the sorbent, 2) loading solution containing the analyte is percolated through the solid phase, the analytes are retained on the sorbent, 3) sorbent is then washed to remove impurities, and 4) the analyte is collected during this elution step.**

## 1.2.5 Micro-solid phase extraction (SPME)

The SPME technique does not use organic solvents, and is practical and economical. It is a solventless alternative to conventional sample extraction techniques. The amount of analyte extracted by the coating at equilibrium is determined by the magnitude of the partition coefficient of the analyte between the sample matrix and the coating material. Analytes are concentrated on the fiber and are rapidly delivered to the column, resulting in improved detection and resolution. SPME is more selective than other methods in that it takes full advantage of differences in the extraction-phase/matrix. Exhaustive extraction can be achieved in SPME when the distribution constants are large enough. In exhaustive extraction, selectivity is sacrificed to obtain a quantitative transfer of target analytes into the extraction phase [69].

Some parameters considered in SPME for development and optimization in this method are: the extraction conditions (including fiber coating, extraction mode, extraction time, agitation method, and sample volume), matrix modifications (pH, ionic strength, sample dilution, organic solvent content, analite derivatisation, and sample temperature), and the desorption

condition (separation/detection system, SPME interface to analytical instrument, and factors affecting desorption efficiency for GC and LC systems) [70].

The sensitivity of an SPME method largely depends on the correct selection of the fiber layer, with its thickness with respect to the compounds of interest also being important. In general, poly dimethylsiloxane (PDMS) and polyacrylate (PA) are widely used in SPME fibers.

Other materials can be used that offer a better selectivity depending on the polarity and volatility of the analytes to be studied. (e.g. Carboxen/Polydimethylsiloxane (Car/PDMS), Carbowax/Divinylbenzene (CW/DVB), Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS), Carbowax/Templated Resin (CW/TPR), and Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)). Figure 1.10 shows coating selectivity based on component volatilities and polarity. SPME fiber can be used to extract the target analytes directly in the field without collecting the sample. It acts as a "sponge" to the surface concentration of the analyte, and can be transferred to a gas or liquid chromatographs easily. A SPME condition requires careful and consistent performance of optimization for reproducible results. Extraction time has a strong effect on the efficiency of the SPME and will improve by increasing the extraction time. In Figure 1.11, the effects of extraction time of SPME are represented. Temperature is an important factor in optimizing the SPME extraction[60]. The extraction efficiency depends also on several other factors such as the degree of stirring, the addition of salt, the pH of the solution, and the addition of methanol.

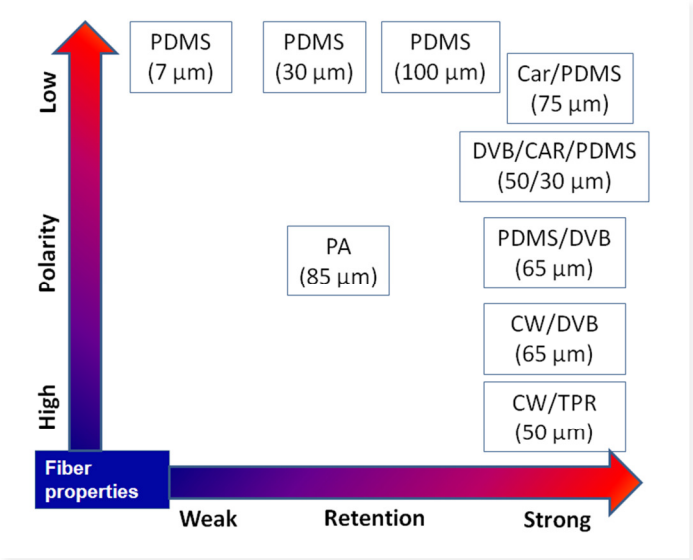



**Figure 1.10 Coating selectivity's based on component volatilities and polarities. Adapted from Kataoka et al. [71].**

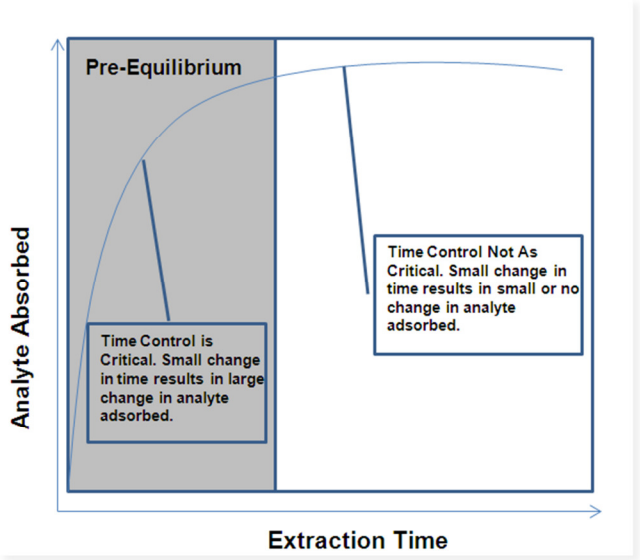



**Figure 1.11 Time effect for SPME extraction. Adapted from Vas et al [72].**

This method has been routinely used in combination with GC and GC–MS, and is successfully applied to a wide variety of compounds. This is true especially for the extraction of volatile and semi-volatile organic compounds from environmental, biological and food samples [71].

Controlled heating of the samples can also affect the extraction in a positive way. However, since the adsorption step is exothermic in nature, the increase in temperature eventually reduces the distribution constant. Therefore, an optimum extraction temperature should be identified. Another parameter that should be studied is the use of agitation during the extraction. Stirring creates contact between the sample and the solvents, which allows for the increase in mass transfer of the analytes. It also minimizes the static liquid layer around the fiber and thus reduces the time to reach the equilibrium. Furthermore the addition of salt or the adjustment of pH can also alter SPME yields. Salt addition increases the extraction efficiency for the more polar and volatile analytes, as it decreases their solubility in the aquatic matrix. Acidic and basic compounds are more effectively extracted under acidic and alkaline conditions, respectively. Consequently, a combination of salt and pH modification often enhances the extraction of analytes.

Finally the addition of methanol prevents the adsorption of the analytes to the glass. On the other hand, the use of methanol causes a competition between the affinity of more non-polar analytes towards the fiber and their affinity towards the solvent. Therefore, an optimum solvent volume should be identified. Figure 1.12 shows the extraction of analytes and, desorption during the injection.

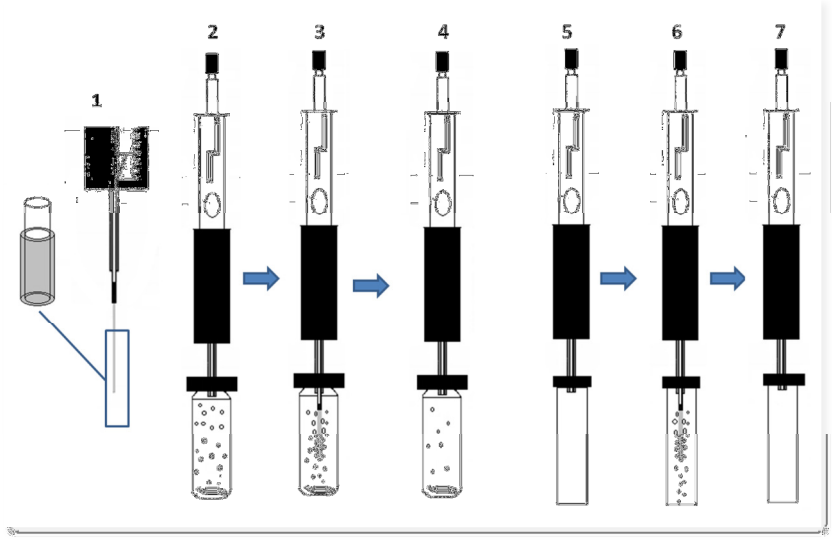



**Figure 1.12 Principles of SPME 1) Retract fiber/withdraw needle and device extraction and, desorption of solid-phase microextraction (SPME) procedure; 2) pierce septum on sample container; 3) expose SPME fiber/extract analytes; 4) retract fiber/withdraw needle; 5) pierce septum in GC inlet, and 6) expose fiber/desorb analytes. Adapted from Augusto et al. and Pawliszyn et al. [69,73].**

Besides the extraction efficiency, desorption step mainly depends on the working temperature of the injection port, the set gas flow and the time of desorption.
All the previous discussed parameters must be kept constant during these analytical steps, as any change can have a tremendous effect on the results.

## 1.2.6 Stir Bar Sorptive Extraction (SBSE)

SBSE was introduced by Baltussen in an analytical practice in 1999 [74]. The stir bar is inserted in an aqueous sample and stirred. The analytes are enriched from aqueous samples by adsorption on a thick film of Poly-dimethyl-siloxane (PDMS) in a cased glass coated magnet. The sample extraction is performed during stirring for a predetermined time. The bar is then removed and placed in a glass tube, which is then transferred to a thermal desorption system, where the analyte is recovered and analyzed by GC or LC. Extraction using sorption is, by nature, a technique of equilibrium. The extraction of analytes from the aqueous phase in the extraction step is controlled by the partition coefficient between the solute and the stationary phase, due to low ratio, high recovery phases are obtained (especially for volatile compounds) [60].

## 1.2.7 Accelerated Solvent Extraction (ASE)

ASE uses conventional solvents at elevated temperatures and pressures to enhance the extraction of organics from solids. The combination of elevated pressures and temperatures

affects the solvent, the sample and their interaction. High pressure also allows the solvents to penetrate deeper into the sample matrix. Additionally, at higher temperatures analyte solubility increases, solvent viscosity and surface tension is reduced, and the mass transfer is faster.

ASE has been applied to the extraction of organic compounds from different samples, and it has also been used as a standard method by the USEPA. It is utilised by the USEPA for the extraction of water insolubles and slightly water soluble, semi-volatile organic compounds (including organophosphorus pesticides OCPs, chlorinated herbicides, PCBs, polychlorinated dibenzodioxinas and polychlorinated dibenzofurens). This is done using environmental samples such as soil, clays, sediments, sludges and waste water.

It is important to note that when extracted from solid samples, ASE yields good recoveries in a short time for the same semi-volatile and non-volatile organic compounds. However, there are drawbacks. ASE generally is not suitable for VOC determination, losses of volatile analytes might occur during the extraction collection step, and the method used in sample preparation is considered to be overly expensive due to the cost of the instrumentation required.

ASE (also called PSE for pressurised solvent extraction) is a relatively new technique that has been applied successfully for the extraction of pesticide residues from various matrices [75]. In practice, a general-purpose solvent is pumped into the extraction of a cell containing the sample, which is then brought to an elevated temperature and pressure. Then, the extract is transferred from the heated cell to a collection tube for cleaning and analysis. The extraction required is almost independent from the mass of the sample and the extraction efficiency depends mainly on process temperature. It is fully automated, and is performed in minutes for quick and easy extraction solvent consumption. ASE could be used as a direct replacement for solvents with intensive techniques such as soxhlet and ultrasound-assisted extraction [60].

### 1.2.8 Soxhlet Extraction (SXE)

Soxhlet extraction is a traditional extraction technique that has been found to be time-consuming and requires large volumes of organic solvents [76]. It has been widely used for organochlorine pesticides, providing efficient extractions which makes it useful as a reference method [77].

Soxhlet is used widely in the extraction of plant metabolites because of its convenience. The main advantage of Soxhlet extraction is that it is a continuous process. As the solvent (saturated in solubilised metabolites) empties into the flask, fresh solvent is recondensed and

extracts the material in the thimble continuously. This makes Soxhlet extraction less time and solvent consuming than maceration or percolation. However, the main disadvantage of Soxhlet extraction is that the extract is constantly heated at the boiling point of the solvent used, and this can damage thermolabile compound and/or initiate the formation of artifacts [78].

## 1.2.9 Ultrasound-Assisted Extraction (UAE)

In recent years, UAE has attracted growing interest because of its effectiveness in the rapid extraction of a number of compounds from food and environmental samples. Its efficiency is comparable to that of classical techniques.

Sonication is the act of applying sound (usually ultrasonic) energy to agitate the particles in a sample for different purposes. When compared to other techniques (such as microwave assisted extraction), UAE uses more affordable equipment and is easier to operate. UAE is used for the extraction of analytes from solid samples using ultrasound radiation in a water bath or with other devices (probes, sonoreactors or microplate horns). The most available and cheapest source of ultrasound irradiation is the ultrasonic bath. However, a more efficient system using a cylindrical powerful probe for the sonication of samples has been developed. The extraction efficiency of a contaminant from a sample by UAE depends on each specific situation, as not all contaminants behave identically. To maximise extraction, it is necessary to optimise different factors, such as the types of solvents used or the irradiation conditions involved (temperature and amplitude of sonication). Other parameters that influence extraction efficacy are: sonication time, sample particle size, sample amount and the ultrasound device employed [79].

UAE is recognized as an efficient extraction technique that dramatically reduces working times, increases yields, and enhances the quality of the extract [80].

## 1.2.10 Microwave-Assisted Extraction (MAE)

The MAE method, which uses microwave energy as a heating source, was introduced in 1986. The use of organic solvents to extract organic pollutants from solids was discontinued in this method. Solutions in this procedure reach boiling point very quickly, resulting in very short extraction times. It is highly efficient compared to conventional methods. In the MAE, the temperature and nature of solvent extraction greatly affect the partitioning of the analytes from the sample matrix into the solvent. The optimization studies in this method usually include such variables as the composition of the solvent, the volume of solvent, the extraction temperature, extraction time, and the characteristics of the matrix (including water content).In order to heat a solvent (or solvent mixture), part of it must be polar (examples

include methanol, ethanol and water). In the case of non-polar solvents with low dielectric constants, substances called sensitizers are added. Sensitizers are molecules that preferentially absorb microwave radiation and give it to other molecules. Although hexane and toluene are potential solvents for MAE, selectivity and extraction efficiency can be modulated by the addition of small amounts of acetone or methanol. MAE is more effective and less expensive them many of the new methods, and it reduces substantially the sample preparation time and solvent volumes compared with conventional extraction techniques.

The amount of solvent used is sufficient enough to ensure solid matrix is completely submerged. However, contrary to conventional extraction techniques, a higher proportion of solvent volume mass of the solid matrix can reduce recoveries in the MAE method. This results in a significant reduction in the use of solvents (usually just 40 mL, compared with 150-200mL in Soxhlet and LLE) [60].

MAE has been mainly used for extracting persistent organic pollutants (like polychlorinated biphenyls or polyaromatic hydrocarbons) from a variety of matrices, such as soils and sediments. For analysis in animal and plant tissues, different analytical methods have been used for pollutants like imidazolinones, organophosphates, organochlorines, pyrethroids, ethylene-bisdithiocarbamates (EBDCs) and other miscellaneous pesticides [81].

### 1.2.11 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) has been used since the 1980s. Several solvents can be used in a supercritical state to extract analytes from different matrices. These include nitrous oxide, pentane, carbon dioxide and ammonia. Unfortunately all have security problems, such as high reactivity and flammability except carbon dioxide. Environmental incentives for the use of supercritical fluids include their inertness to most materials, their non-toxicity, and the fact that they generate minimal solvent waste. The physical properties of supercritical fluids provide several advantages over liquid. Supercritical fluids diffuse through solids like gases but dissolve analytes like liquids. These results in the extraction rate are enhanced, as well as their less thermal degradation. These units offer quick movements (balance) like a gas, but salvation or solubilisation like a liquid [60].

The unique properties exhibited by supercritical fluids means that it has already been applied to the analysis of pesticide residues in solid samples. SFE is selective and less-solvent-consuming, thus it is environmental friendly. The most serious problem of off-line SFE methods is the occurrence of evaporation in the collection of the solvent at the end of extraction to acquire high pre-concentration factor. Additionally, this procedure is time-

consuming and contaminates the environment and collected analytes may be lost or degradated [63].

### 1.2.12 Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)

A recent original analytical methodology was developed combining the extraction/isolation of pesticides from food matrices and extract cleanup [82]. They joined the acronym QuEChERS for it, i.e. Quick, Easy, Cheap, Effective, Rugged and Safe. This technique involves micro scale extraction using acetonitrile, as well as the purifying of the extract using dispersive solid-phase extraction (d-SPE). Since the development and publication of this method, QuEChERS has been gaining significant popularity. It is the method of choice for food analysis because it combines several steps, and it extends the range of pesticides recovered compared to other extraction techniques. It has undergone various modifications and enhancements over the years since its first introduction [83].

Other matrices such as biological [84] and environmental samples, including soils [85], are also studied and continually analyzed by this technique. Although QuEChERS has mainly been used for the determination of pesticides in soils, other compounds, like pharmaceuticals [84], β-lactam antibiotics [86], and veterinary drugs [87-89], have been determined using QuEChERS. The versatility of QuEChERS has been demonstrated by its acceptance outside of its traditional application areas. It is most often used for pesticide residue analysis as described, but more recently has expanded its scope to other trace contaminants [90,91]. QuEChERS methodology reduces sample size and quantities of laboratory glassware. The QuEChERS method requires fewer steps, which means that (no blending, filtration, large volume quantitative transfers, evaporation/condensation steps, solvent exchange is required): This is very significant, as every additional analytical step complicates the procedure and is also a potential source of systematic and random errors. For determining pesticide residues in food matrices, the usual solvents have been acetone, ethyl acetate, and acetonitrile. All of these solvents ensure large analyte recoveries. It is important to note that acetone is readily miscible with water, but the separation of water from this solvent is impossible without the use of non-polar solvents. On the other hand, ethyl acetate is only partially miscible with water, which renders superfluous the addition of non-polar solvents to separate it from water. Additionally, the most highly polar pesticides do not separate in it. Acetonitrile extracts of different matrixes contain fewer interfering substances than the corresponding ethyl acetate and acetone extracts, and acetonitrile can be separated fairly easily from water (salting out).

Therefore, acetonitrile it is the extraction solvent of preference in the QuEChERS methodology [83]. Figure 1.13 shows the steps of extraction of the analytes.

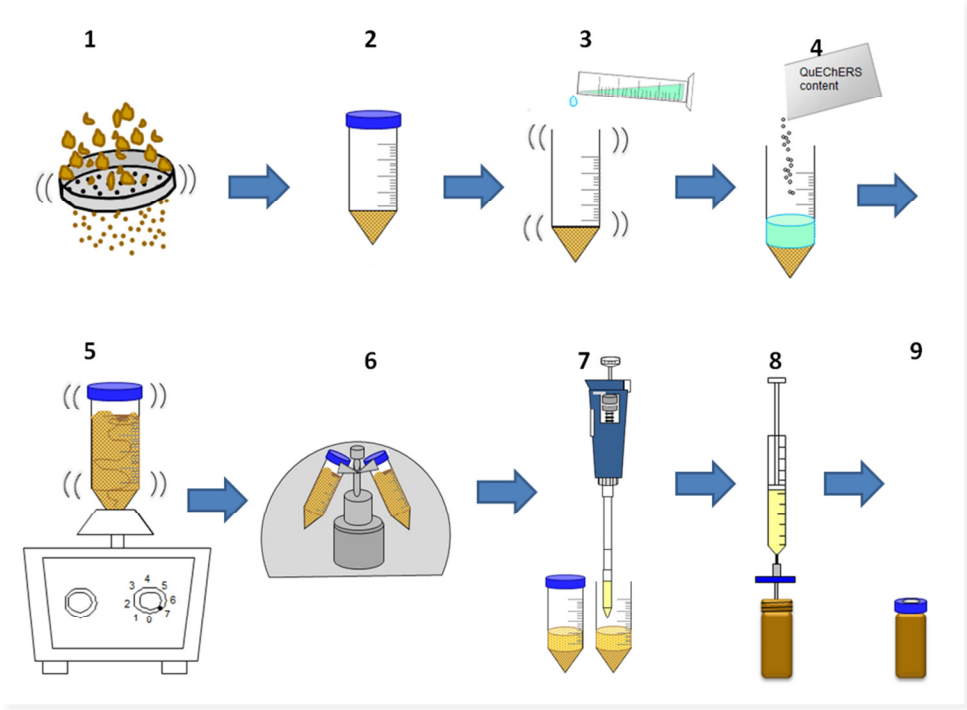



**Figure 1.13 Step in QuEChERS extraction. 1) Sieving, 2) teflon tube with soil sample, 3)addition of the extraction solvent and hand mix, 4) addition of the QuEChERS content, 5) vortex, 6) centrifugation step, 7) aliquot of the supernatant, 8) filtration with a syringe filters, and 9) vial with the extract to analysis. Adapted from Vera et al. [92] .**

To the optimization of the extraction parameters briefly describe some of the most important aspects such as; 1) hydration step, 2) ratio sample/volume, 3) extraction solvent (type, volume and pH), 4) QuEChERS content, extraction time and homogenization technique, 5) prevention of agglomeration, 5) clean-up.

In first step is hydration step, the addition of water to the sample prior to the QuEChERS extraction is used to weaken interaction  ns of the analytes within the matrix [93]. For ratio sample/volume, typically the best way to improve efficiency of an analytical method is to reduce sample size to the minimum amount and scale the method accordingly [82]. Higher sample weights or larger solvent volumes will compromise a proper homogenization due the capacity of the centrifuge tube [94]. Extraction solvent is an aspect that has to be considered, including: the ability to cover the desired analytical spectrum (ranging from the polar to the non-polar compounds); the selectivity that can be reached during extraction; partitioning and clean-up; achieving separation from water; amenability to chromatographic separation techniques; cost; safety; environmental impact; and, handling concerns (e.g., ease of

evaporation, volume transfers) [94]. QuEChERS Content, the initial single-phase extraction with ACN is followed by the addition of salts ($MgSO_4$ and NaCl) to induce phase separation. The addition of NaCl typically leads to increased recoveries of polar compounds, but this also depends on the nature of the solvents involved in the partitioning step, and allows the control of the percentage of water in the organic phase. The use of $MgSO_4$ also has the ability to bind large amounts of water and thus significantly reduce the water phase. This also promotes partitioning of analytes into the organic layer. Nevertheless, to bind a significant fraction of water, $MgSO_4$ should be added at amounts well exceeding its saturation in water [94,95]. The AOAC 2007.01 method uses an acidification of the extraction solvent with 1 % acetic acid. The addition of an anhydrous ($CH_3COONa$) buffer, to protect the base sensitive analytes from degradation, provides superior recovery for pH sensitive compounds [96]. The European Norm EN 15662 includes citrate buffering reagents that preserve base sensitive analytes [97]. Prevention of agglomeration is other factor important. This can occur even with vigorous homogenization, and can compromise the extraction [93]. QuEChERS suppliers have prescribed the use of ceramic pieces to break up salt agglomerates to facilitate sample homogenization. To avoid the formation of agglomerates, these authors added the QuEChERS content slowly and continuously with slow vortexing [90]. In the case of clean-up typically involve mixing an aliquot of the sample extract with a small amount of sorbent, thus making the clean-up process easier [91].

## 1.3 Chromatographic determination of Pesticides in environmental samples

The gas chromatography provides a time separation of components in a mixture. The basic operating principle of a gas chromatograph involves the volatilization of the sample in a heated inlet port (injector), separation of the components of the mixture in a specially prepared column, and detection of each component by a detector [98]. Chromatography is one of the main techniques in the determination of multi-residue, making it ideal for determining pesticides analysis methods. Analytical separative techniques such as gas chromatography (GC) or liquid chromatography (LC) are necessary, and can be associated with a wide variety of selective detection methods. GC has certain advantages when volatile and semi-volatile compounds are involved [99-106]. The most commonly used GC detectors are the following: element-selective detectors (such as the Electron-Capture Detector (ECD)), which are used for the detection of chlorinated pesticides; the Nitrogen Phosphorus Detector (NPD), used mostly for the detection of nitrogen containing pesticides; and the Flame Photometric Detector (FPD), which is used for the detection of organophosphorus pesticides.

Since the early 1970s most routine pesticide residue analysis has been conducted by gas chromatography (GC). This is done in combination with electron capture, nitrogen-phosphorous, and/or flame photometric detection. It is also important to note that GC-MS use is steadily increasing, especially for confirmation and identification[4,101,107].

## 1.3.1 Gas chromatography- Electron Capture Detector (GC-ECD)

With an ECD, a beta emitter such as tritium or $^{63}Ni$ is used to ionize the carrier gas. Electrons from ionization migrate to the anode produce a steady current. If the GC effluent contains a compound that can capture electrons, the current is reduced, as resulting negative ions move more slowly than electrons [108]. Consequently, the loss of electrical current is the signal measured. The ECD is very sensitive to materials that readily capture electrons. These materials frequently have un-saturation and electronegative substituent. Because the ECD is sensitive to water, the carrier gas must be dry [98].

## 1.3.2 Gas chromatography-mass spectrometry (GC-MS)

An interface in GC-MS is a device for transporting the effluent from the gas chromatograph to the mass spectrometer. This must be done in such a manner that the analyte neither condenses in the interface nor decomposes before in enters the mass spectrometer ion source [98]. For capillary columns, the usual practice is to insert the exit end of the column into the ion source. This is possible because under normal operating conditions the mass spectrometer pumping system can handle the entire effluent from the column. It is then only necessary to heat the capillary column between the GC and the MS ion source. This is done to eliminate cold spots where analytes could condense. The interface must be heated above the boiling point of the highest-boiling component of the sample [98].

A mass spectrometer is an instrument that measures the mass-to-charge ratio (m/z) of gas phase ions and provides a measure of the abundance of each ionic species. The measurement is calibrated against ions of known m/z. In GC-MS, the charge is almost always 1 so that the calibrated scale is in Daltons or atomic mass units. All mass spectrometers operate by separating gas phase ions in a low pressure environment by the interaction of magnetic or electrical fields on the charged particles. The most common mass spectrometers interfaced to gas chromatographs are the so-called quadrupole and magnetic-sector instruments [98].

Nowadays, using GC combined with MS, simultaneous determination and confirmation of pesticide residues can be obtained with one instrument in one analytical run [109]. The most widely used and recommended confirmatory technique for pesticide residue analysis has

been the MS with electron ionization (EI). Electron Impact ionization is by far the most commonly used ionization method. The effluent from the GC enters a partially enclosed ion source. Electrons boiled from a hot wire or ribbon (filament) are accelerated typically by 70 V (and thus have 70 eV of energy), then are entered into the ion source through a small aperture. When these electrons pass near neutral molecules, the impact may create sufficient energy to remove outer shell electrons, producing additional free electrons and positive (molecular) ion. The energy imparted by this type of ionization is high and, with only rare exceptions, causes part or all of the molecular ions to break apart into neutral atoms and fragment ions. This ionization technique produces almost exclusively positively charged ions. The technique of chemical ionization (CI) is a direct outcome of fundamental studies of ion/molecule interactions. Over the past 10 years, there has been an increased interest in negative ion/molecule reactions as an ionization technique in the field of Negative Chemical Ionization Mass Spectrometry (NCI-MS). An NCI process is a low energy process with limited fragmentation (easily identifiable molecular ion), and providing simple mass spectra in comparison to the electron impact ionization (EI) technique [107]. In Figure 1.14 analytes in Single Ion Monitoring (SIM), scan in MS mode inside of an Ion Trap (IT) are shown.

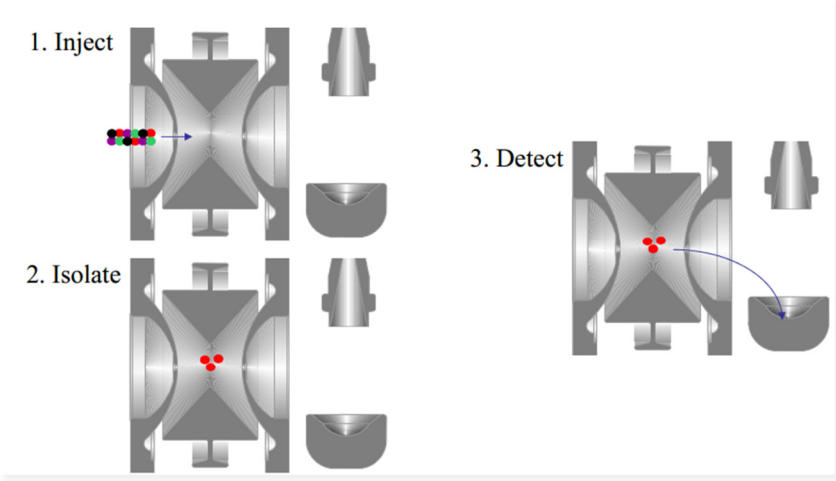



**Figure 1.14 Inject and detect SIM scan MS. Adapted from Hubschmann[110].**

## 1.3.2.1 Mode injection

There are four basic types of injection techniques: isothermal (hot), split and splitless, on-column, and programmed temperature vaporization (PTV). The isothermal split and splitless injections are performed in the same inlet called a split/splitless inlet. This split/splitless inlet is known for its simplicity and robustness. However, in most cases the classic splitless injection only enables 1–2µL of a liquid onto capillary columns. Depending on the solvent,

this injection can be increased up to 5–10µl using a pressure pulse during the sample introduction process.

In the split injector, the injected sample is vaporized into a stream of carrier gas, and a portion of the sample and solvent (if any) is directed onto the head of the CG column. Normally, 1-2 µl of sample is injected into a split -type injector, but larger volumes (3-5 µl) can also be used [98].

Splitless Injection is the most frequently applied injection technique. However, some adverse effects such as discrimination of low volatiles, sorption, and thermo degradation can occur. Significant suppression of these effects in the injection port is achieved by the application of pressure pulse during the splitless period (pulsed splitless injection) [111]. In the splitless injection, the splitter vent is closed so that the entire sample flows onto the head of the column. After a specific time, called the purge activation time, the splitter vent is opened to purge solvent from the injector and from any low-boiling components of the sample that are not adsorbed by the column. In the splitless injection method the majority of the sample is placed onto the head of the cool column and purges most of the volatile solvent. For this reason, and because large amounts of sample can be injected, splitless injection is used for trace analysis. The splitless method is not recommended for wide-boiling range samples if quantification is required. For best results, the solvent boiling point should be at least 20 ºC below the lowest boiling component of the sample. Although splitless injection is the preferred method for trace analyses, it is worth noting that it requires optimization of parameters such as column temperature and purge time [98].

For the injection of large volumes of up to hundreds of microliters of sample, on-column and PTV injection techniques have mainly been used (and/or modified). The most critical problem in Large Volume Injection (LVI) is huge solvent vapor volumes resulting from the expansion of the large liquid volume of the injected solvent. On-column injection solves this problem using a retention gap, which provides room for the large injected solvent volume to condense and expand. The PTV injection separates solvent vapor from analytes through venting of the vapor in the liner [112].

With on-column injection, the sample is injected directly onto the column using a small syringe needle. This technique is easier to use with larger bore GC columns, but modern gas chromatographs can precisely control the on-column injection process. This includes the automatic control of heating and cooling of the injector. This method of analysis gives good quantitative results and is especially valuable for wide-boiling ranges and thermally labile samples. With this technique, a short section of uncoated (inert) fused silica capillary tubing is often inserted between the injection port and the capillary analytic column [98].

# CHAPTERS 2

# MASS SPECTROMETRY PARAMETERS OPTIMIZATION FOR THE PESTICIDES DETERMINATION IN *n*-HEXANE WITH GAS CHROMATOGRAPHY ION-TRAP TANDEM MASS SPECTROMETRY

# CHAPTER 2 Mass Spectrometry parameters optimization for the pesticides determination in *n*-hexane with Gas chromatography ion-trap tandem Mass Spectrometry *

## 2.1 Introduction

The multi-residue methodologies that can determine a large number of pesticides simultaneously with satisfactory sensitivity and selectivity are highly required [113]. Stringent international safety standards require monitoring some chemicals in water, food and biological samples at trace level. Gas chromatography coupled to an electron capture detector (GC-ECD), a nitrogen phosphorus detector and different other detectors are still widely used as analytical technique for the trace analysis of pesticides in various environmental and food matrices with high sensitivity [50,114-122]. Trace analyses are being developed for more complex and dirty samples in which sample preparation is a key element, and fast gas chromatography (GC) and various combinations of GC and liquid chromatography (LC) with mass spectrometry (MS) are becoming more important [123].

Ion trap (IT) GC-MS has the potential to identify analytes at trace levels and to avoid the influence of matrix components as well as allows for selective analysis by MS/MS. This could be achieved by collision-induced dissociation (CID) of a selected unique precursor ion that produces ions in sufficient abundance ratios specific to the detection of a particular molecule [124]. In order to attain high sensitivity to achieve low detection limits, the instrumental parameters of IT-MS affecting the performance of this system must be thoroughly optimized [125]. Although parameters for some compounds are available in MS/MS libraries, the optimization of GC–MS/MS parameters is indispensable in order to run with best efficiency [126-129]. There are a few papers describing the optimization and the best parameters for each analyte in a given apparatus [130]. The optimization of GC-MS/MS system requires assessing IT-MS parameters influence by the approach of changing one-factor-at-a-time (OFAT) [130].

*Adapted from: Virgínia C. Fernandes, Jose L. Vera, Valentina F. Domingues, Luís M. S. Silva, Nuno Mateus, Cristina Delerue-Matos, Mass Spectrometry Parameters Optimization for the 46 Multiclass Pesticides Determination in Strawberries with Gas Chromatography Ion-Trap Tandem Mass Spectrometry, Journal of The American Society for Mass Spectrometry, Volume 23, Issue 12, pp 2187-2197, 2012

This approach does not give information on interactions between factors, so it can miss the optimal settings when interactions do occur. Statistically designed experiments such as Plackett–Burman and central composite designs can help to optimize analytical parameters much more efficiently and in less experimental runs [128]. Optimization of MS/MS parameters is a hard work for each analyte; hence, knowledge of optimal values will save considerable time in analytical method development.

The aim of the present study was to establish an overall analytical method and optimization of a set of instrumental parameters in order to attain the highest possible sensitivity for pesticides determination.

Particular attention was paid to the optimization of five IT-MS parameters, namely, the duration of the ion isolation waveform voltage [isolation time (IT)], the duration of the ion excitation [excitation time (ET)], the mass range window around the ion of interest [isolation mass window (IMW)], excitation voltage (EV) and the maximum excitation energy (q), which is defined as the amount of energy that holds a precursor ion in the ion trap during excitation.

## 2.2 Experimental Part

### 2.2.1 Chemicals and solvents

Reference standards were purchased from Sigma Aldrich, Riedel-de Häen and Chem Service. *n*-hexane and methanol were chromatographic grade and were supplied by Merck (Darmstadt, Germany).

### 2.2.2 Standard solutions preparation

Stock standard solutions (approximately 2000 μg $L^{-1}$) were prepared by dissolving reference standards in *n*-hexane and methanol and were stored in a freezer at 4 ℃. Working pesticide standard mixtures were prepared by dilution of stock solutions in *n*-hexane.

### 2.2.3 Apparatus

GC-MS/MS instrument, TRACE GC Ultra (Thermo Fisher Scientific, Austin, TX, USA) gas chromatograph coupled with a Polaris Q ion trap mass spectrometer was used.

The system included an AS-3000 autosampler. A ZB-XLB capillary column from Phenomenex (30 m×0.25 mm×0.25 μm) was used for chromatographic separation. The system was controlled by Xcalibur software, ver. 1.3.

## 2.2.4 Gas Chromatograph Conditions

The column oven temperature was programmed as follows: initial temperature 40 °C (held for 1 min), increased by 30 °C $min^{-1}$ to 220 °C (held for 5 min), increased by 10 °C $min^{-1}$ to 250 °C and held at this temperature for 20 min and finally increased again by 5 °C $min^{-1}$ to 285 °C and held at this temperature for 5 min. The mass spectrometer was operated in electron ionization mode at 70 eV with an external ionization source. The inlet temperature was 240 °C and helium (purity≥99.999 %) was used as carrier gas at 1 mL $min^{-1}$ and the injection volume was 2 µL in the splitless mode. The interface line and ion source temperature was 250 °C and the electron multiplier was operated at 2100 V (autotune to gain of $1\times10^{7}$).

MS/MS conditions such as isolation (wideband application (IMW), isolation time (IT)), fragmentation (excitation time (ET) and voltage (EV) and factor "q" were optimized for each analyte, beginning with the following base conditions: factor q=0.45, EV=1 V, IT=12 ms, ET=15 ms, IMW=1 and carrier flow 1.3 mL $min^{-1}$.

## 2.3 Limit of Detection

The limit of detection (LOD), also defined as the lowest concentration that the analytical process can differentiate from background levels, was estimated for a signal-to-noise ratio (S/N) of three from the chromatograms analysis.

## 2.4 Multiple Linear Regression Analysis

For each pesticide, multiple linear regression tests were conducted in order to estimate the relationship between the statistically significant parameters of IT-MS/MS detection and the instrumental signal response by fitting a linear equation to observed data.

## 2.5 Macro Edition in Microsoft Excel

A visual basic for macro applications (VBA) was developed in Microsoft Excel in order to assess the maximum of 8 ET × 6 IT × 5 EV × 3 q × 3 IMW combinations, available in the IT-MS/MS detector, based on the predicted value determined by the multiple regression equation.

## 2.6 Results and Discussion

The optimization of MS/MS parameters in IT-MS was carried out in four steps: (1) isolation of precursor ion and subsequent product ion selection, (2) screening analysis, (3) multiple linear regression test to check the importance of each parameter on the signal response, fitting data to linear and second order models for the significant parameters and model discrimination, and finally (4) combinatorial optimization based on the best previously chosen model.

### 2.6.1 Isolation of Precursor Ion and Product Ions Selection

The most abundant ion from the spectra of the different pesticides was selected as precursor ion and it was then isolated in the ion trap and fragmented by collision induced dissociation (CID). The two most abundant product ions, Q1 and Q2 [113] were selected and monitored and the spectrum was obtained with the default operating parameters from the GC-MS/MS system (IT = 12 ms, ET = 15 ms, IMW = 1, q = 0.45, EV = 1.0 V) Table 2.1.

**Table 2.1 Precursor ions and the products ions (Q1 and Q2) for the selected pesticides.**

| Chemistry class | EDPs | RT (min) | CAS | Ion precursor | Q1 | Q2 |
|---|---|---|---|---|---|---|
| **Chloroacetanilide** | alachlor | 10.92 | 15972-60-8 | 188 | 160 | 132 |
| **Dicarboximide** | vinclozolin | 11.03 | 83792-61-4 | 212 | 172 | 145 |
| **Pyrethroids** | β-cyfluthrin | 28.37 | 68359-37-5 | 163 | 91 | 127 |
| | λ-cyhalothrin | 21.53 | 91465-08-6 | 181 | 152 | 141 |
| | cypermethrin | 29.49 | 52315-07-8 | 181 | 163 | 152 |
| | fenpropathrin | 19.27 | 64257-84-7 | 181 | 152 | 153 |
| | fenvalerate | 35.66 | 51630-58-1 | 125 | 121 | 132 |
| | Permethrin | 24.69 | 52645-53-1 | 183 | 165 | 153 |
| **Triazines** | atrazine | 9.62 | 1912-24-9 | 200 | 164 | 122 |
| | atrazine desethyl | 8.87 | 6190-65-4 | 172 | 136 | 145 |
| | simazine | 9.56 | 122-34-9 | 200 | 172 | 164 |

### 2.6.2 Screening Analysis

Different values of EV, ET, IMW, q, and IT were set up by a one-factor-at-a-time (OFAT) method, meaning that when one parameter is changed the others are fixed at their default value. Regarding multiclass pesticides signal maximization, the search of the best IT-MS parameters is necessary. Thus, the parameters were varied in order to determine the suitable values at which both absolute peak area of the product ions and signal to noise (S/N) ratio were maximized for the 11 pesticides under investigation. The concentration of

the standard mixture solution used in this work was 150 µg $L^{-1}$. An important increase of area was observed when "q" factor increases from 0.225 to 0.45. This increase is more remarkable for vinclozolin. In particular for vinclozolin achieved a LOD of 0.76 µg $L^{-1}$ for q=0.225 and LOD of 0.39 µg $L^{-1}$ for q=0.45 respectively. In the Figure 2.1 shows a variation in signal response of the 11 pesticides as a result of alteration of the following parameters, IMW, IT, EV and ET. It was more pronounced for alachlor (IMW=4), and vinclozolin (IMW=1) as a result of IMW. In the case of IT, the variation in signal response was more pronounced for vinclozolin (IT=12). The S/N value for vinclozolin showed a maximum for IT=12 ms. As to EV dependence, the pesticides with higher variation in signal to noise value were vinclozolin (EV=1), and alachlor (EV=0.2 and EV=0.5). Vinclozolin revealed a random variation with respect to ET influence. Hence, for some pesticides it seems that better LODs can be achieved by changing the IMW to detect neighboring ions or by varying other parameters of the ion trap (i.e. IT, ET, EV or q). This screening analysis proves that the variation of one parameter can mean a change in the response, so the importance of applying statistical studies may allow the prediction of the best parameter conditions.

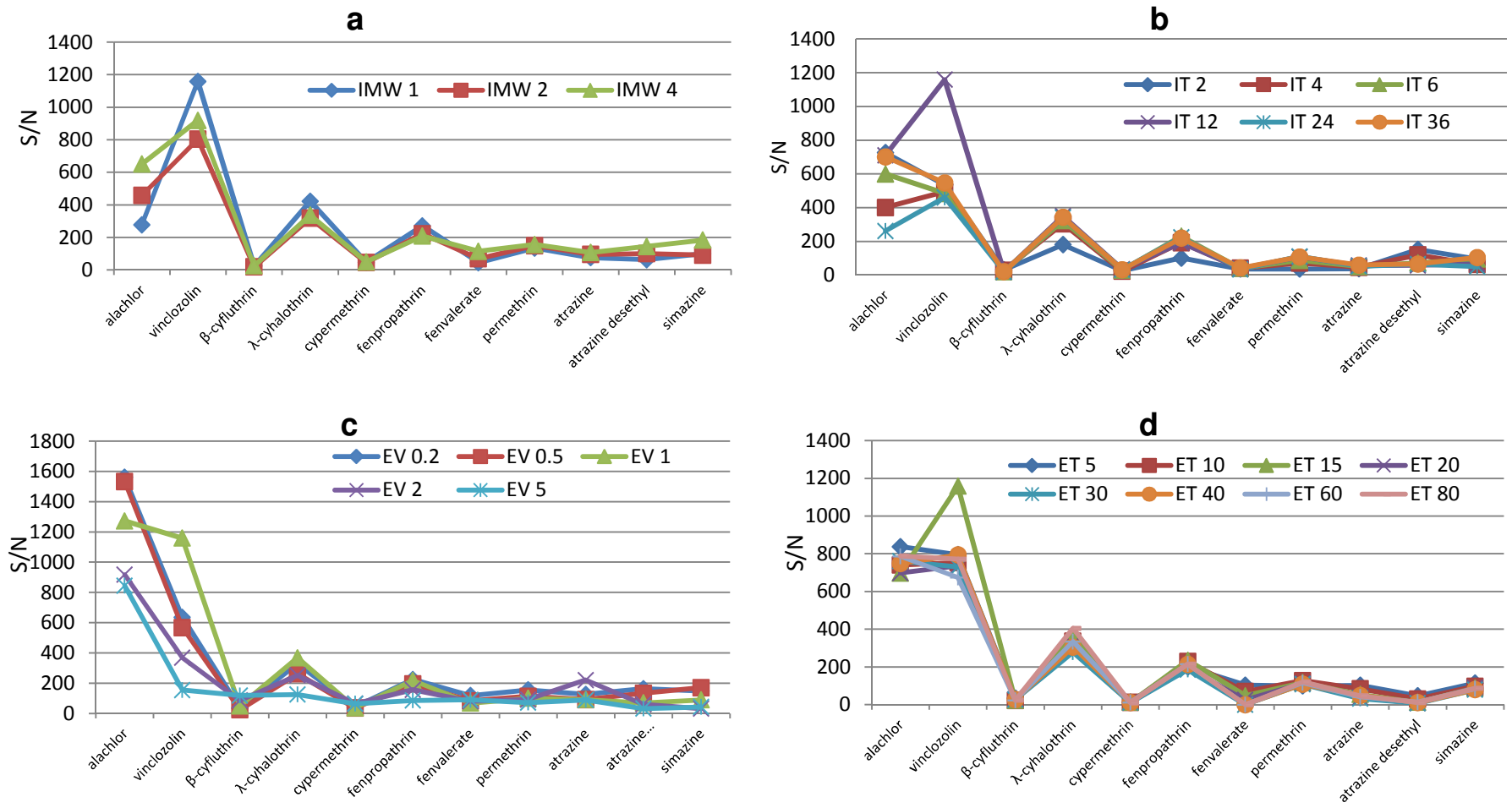



**Figure 2.1 Plots of average signal to noise (S/N) versus a) IMW, b) IT, c) EV, and d) ET.**

## 2.6.3 Multiple Linear Regression

Multiple linear regression tests were carried out in order to estimate the influence of each parameter up to five on the IT-MS analytical response. In most cases, the true functional relationship is unknown. This study allowed finding the parameters that have significant influence on signal response. Hence the IMW, IT, ET, EV, and q values were screened in order to discard the irrelevant parameters keeping the remaining for subsequent optimization.

## 2.6.4 Statistical evaluation

## 2.6.4.1 Model Discrimination

To evaluate the "best" empirical regression equation, the analytical responses obtained were fitted to linear models that included only first order terms (Equation 1), and both first and second-order (curvature) terms (Equation 2). The regression equations for the analytical response (Y) in terms of the factors ($x_i$) are as follows:

$$First\ order: Y = \beta o + \sum_{i=1}^{n} \beta_i x_i \qquad \text{Equation 1}$$

$$Second\ order: Y = \beta o + \sum_{i=1}^{n} (\beta_i x_i + \beta_{ij} u x_i^2) \qquad \text{Equation 2}$$

where $\beta_0$ is the independent coefficient; n refers to the number of significant factors to a maximum of 5, $x_i$ are the values of IT-MS parameters (factors), $\beta_i$ represent the coefficients for the main effect; and $\beta_{ij}$ are the coefficients for the second order effect where *i*=1,2,3,4 and 5 stands for IMW, IT ET, EV, and q, respectively.

## 2.6.4.2 Test for Significance of Regression

The test for significance of regression is a statistical test that checks if there is a linear relationship between the response variable y and a subset of the regressor variables $x_1$, $x_2$,…,$x_k$. The test procedure involves an analysis of variance partitioning the total sum of squares $SS_T$ into a sum of squares due to the model (or to regression) and a sum of squares due to the residual (or error), say :

$$SS_T = SS_R + SS_E \qquad \text{Equation 3}$$

Based on the theory for discrimination of nested models described in textbooks[131,132], a comparison between first and second order models was performed. A kind of a likelihood test

ratio adapted for regression models was conducted. For this, a statistic T was computed as follows:

$$T = \frac{SS_{E1} - SS_{E2}}{SS_{E2}} * \frac{n - k_2}{k_1} \qquad \text{Equation 4}$$

for n data points, where k is the number of model coefficients and subscripts *1* and *2* refer to first and second order model, respectively. This ratio obeys to an F distribution, asymptotically for large n, with $k_1$ degrees of freedom in numerator and n-$k_2$ degrees of freedom in denominator. For T values greater than $F_{critical}$ the null hypothesis that states that there are no differences between the two models must be rejected. It was proven that the second order model fits the experimental data better in most of the cases with a level of significance of 0.05 as presented in Table 2.2. This fact implies the existence of stationary points for the model equation because of the curvature of the surface response. At the same time, the coefficients of determination ($R^2$) were computed for the two models and are also presented in Table 2.2. Although the second order model always achieved higher $R^2$ values, the regression test proves that the ones marked by an asterisk in Table 2.2 are not significantly better.

**Table 2.2 Coefficient of Determination for First and Second Order and Statistic T Used for Model Discrimination**

| Pesticides | $R^2$ | $R^2$ | Regression test |
|---|---|---|---|
| | First order | Second order | T (Fcritical) |
| **alachlor** | 0.7353 | 0.9309 | 14.64 (2.41) |
| **vinclozolin** | 0.5823 | 0.6084 | 0.34 (2.41)* |
| **β-cyfluthrin** | 0.3970 | 0.5919 | 2.47 (2.41) |
| **λ-cyhalothrin** | 0.7051 | 0.8915 | 8.88 (2.41) |
| **cypermethrin** | 0.6629 | 0.7695 | 2.39 (2.41)* |
| **fenpropathrin** | 0.7139 | 0.9092 | 11.11 (2.41) |
| **fenvalerate** | 0.5954 | 0.9042 | 16.66 (2.41) |
| **permethrin** | 0.7417 | 0.8520 | 3.85 (2.41) |
| **atrazine** | 0.5754 | 0.8770 | 12.67 (2.41) |
| **atrazine desethyl** | 0.3382 | 0.7134 | 6.76 (2.41) |
| **simazine** | 0.6254 | 0.8886 | 12.20 (2.41) |

*Second order model is not significantly better than first order model

## 2.6.4.3 Tests on Individual Regression Coefficients

The chosen model might be more accurate with the deletion of one or more of the coefficients belonging to the general Equations 1 or 2. The statistics for testing the significance of any individual regression coefficient is:

$$t_0 = \frac{\hat{\beta}}{\hat{\sigma}^2 C_{jj}} \qquad \text{Equation 5}$$

where C*jj* is the diagonal element of the matrix variance covariance $(X^TX)^{-1}$ for the corresponding $\hat{\beta}_j$. The denominator of the Equation 5 is often called the standard error of the regression coefficient $\hat{\beta}_j$, that is:

$$se(\hat{\beta}_j) = \sqrt{\hat{\sigma}^2 C_{jj}} \qquad \text{Equation 6}$$

where $\hat{\sigma}$ refers to the standard error of regression calculated as:

$$\hat{\sigma} = \sqrt{\frac{SS_E}{n-k}} \qquad \text{Equation 7}$$

The IMW is the mass range window around the ion of interest that is isolated for optimization of MS/MS parameters. The multiple regression test proved that the IMW parameter has significant effect in the area response of vinclozolin, λ-cyhalothrin, fenpropathrin, and permethrin. The IMW parameter is not significant for other pesticides studied.

Another important parameter for MS/MS optimization is the maximum excitation energy (q), which is defined as the amount of energy that holds a precursor ion in the ion trap during excitation and thus influences the usable mass range. Only three values (instrument characteristics) can be set for “q” The area response related to q=0.45 is 4.95, 4.68 times higher than with q=0.225 for fenpropathrin, and λ-cyhalothrin respectively. Since higher “q” values allow more energy to be deposited in the precursor ion before its dissociation (CID), it can be concluded that these pesticides need more energy for fragmentation. Regarding multiple linear regression test, “q” has a significant effect on the signal response for most of the fenpropathrin, λ-cyhalothrin, cypermethrin (for q= 0.45) and bifenthrin, permethrin (q= 0.30).

The isolation time (IT) is defined as the duration of the ion isolation waveform voltage applied to isolate a selected precursor ion. An initial value of 12 ms was imposed, which was afterward changed for optimization purposes of the S/N ratio of individual compounds. The test on individual regression coefficients showed that more than half of the studied pesticides were affected by the variation of this parameter.

As can be proven in the multiple linear regression tests, the excitation time (ET) parameter has effect on the MS/MS determination of about 54 % of the pesticides tested (atrazine-desethyl, atrazine, vinclozolin, fenpropathrin, λ-cyhalothrin, and fenvalerate). The results showed that an optimum ET parameter is achieved with a value of 5 ms for the different

pesticide. The EV was significant parameter for 72 % (atrazine-desethyl, simazine, atrazine, vinclozolin, fenpropathrin, λ-cyhalothrin, permethrin, and fenvalerate), and IT 54 % (atrazine-desethyl, atrazine, vinclozolin, permethrin, β-cyfluthrin, and fenvalerate) of the studied pesticides.

In summary, the statistical analysis allowed a quick evaluation of the variation in the signal response of certain pesticides as a result of ion trap parameters change.

The obtained optimum conditions for the instrumental parameters were used to check the sensitivity of IT-MS. Due to the absence of background signals, high signal to noise (S/N) ratios were sought.

### 2.6.4.4 Combinatorial Optimization

The aim of fitting experimental data to a regression model was to predict the chromatographic signal response for other IT-MS parameter combinations that were not tested in the laboratory. When the second order linear model (Equation 2) does not significantly fit data better than the first order one (Equation 1), the optimal matching corresponds to any of the extreme points of all allowed significant parameter values. Conversely, if the second order model fits better but the stationary points are not maxima or even if they are but do not match any possible combination of MS/MS parameters, it becomes necessary to screen all allowed combinations and to calculate the response in order to find the maximum S/N ratio. For this purpose, a combinatorial optimization was carried out. A visual basic for applications macro in Microsoft Excel® environment was created to evaluate the regression model equation for a maximum of 8 ET×6 IT×5 EV×3 q×3 IMW combinations in a total of 2160 arrangements and save the best value. The maximum signal and optimal match of the MS/MS parameters are presented in Table 2.3. Concerning the excitation voltage (EV) concerns, the value that maximizes the response when this parameter is significant was 0.2 V and several others where the EV had no significant effect on the signal response (e.g., β-cyfluthrin). In general, the optimal ET value was 5 ms corresponding to the lowest allowed was obtained. The “q” values that maximize the response were 0.30 and 0.45. It can also be extracted from Table **2.3** that there is a strong correlation between ET and EV parameters. When both parameters are significant there is a linear positive relationship between them.

**Table 2.3 The best MS/MS significant parameter combinations**

| Pesticides | ET (ms) | IT (ms) | EV (v) | q | IMW |
|---|---|---|---|---|---|
| **alachlor** | – | – | – | – | – |
| **vinclozolin** | 5 | 24 | 0.2 | – | 4 |
| **β-cyfluthrin** | – | 2 | – | – | – |
| **λ-cyhalothrin** | 5 | – | 0.2 | 0.45 | 4 |
| **cypermethrin** | – | – | – | 0.45 | – |
| **fenpropathrin** | 5 | – | 0.2 | 0.45 | 4 |
| **fenvalerate** | 5 | 12 | 0.2 | – | – |
| **permethrin** | – | 2 | 0.2 | 0.3 | 4 |
| **atrazine** | 5 | 12 | 0.2 | – | – |
| **atrazine desethyl** | 5 | 12 | 0.2 | – | – |
| **simazine** | – | – | 0.2 | – | – |

## 2.7 Conclusions

The selected optimization strategy based on multiple linear regressions allows for an efficient method development. It is not only helpful to optimize all compounds or a group of them, as it can also help to establish the optimum values for all parameters of the MS/MS system. For pesticides, strategy based on multiple linear regressions which don´t have values, was used the parameter base conditions (factor q=0.45, IT=12 ms, ET=15 ms, EV=1 and IMW= 1).This work shows that the statistics study is a useful tool to optimize this kind of analytical parameters. Particularly, multiple linear regressions were an important tool to predict the "best" combination of IT-MS parameters to maximize the analytical response within the range of values experimentally tested in this work.

# CHAPTERS 3

# OCCURRENCE OF ENDOCRINE DISRUPTING PESTICIDES IN RIVERS, DETERMINATED BY SPME-GC-MS

# CHAPTER 3 Occurrence of endocrine disrupting pesticides in rivers, determinate by SPME-GC-MS*

## 3.1 INTRODUCTION

Chemical pollution of surface water presents a threat to the aquatic environment with effects such as acute and chronic toxicity to aquatic organisms, accumulation in the ecosystem and losses of habitats and biodiversity, as well as a threat to human health [133].

Pollution through the discharge, emission or loss of priority hazardous substances must cease or be phased out in the most economically and environmentally effective manner [134]. Identifying priority hazardous substances has been a main concern of several international institutions based on the precautionary principle, relying, in particular, on potentially adverse effects and on a scientific assessment of the risks.

Regarding pesticides, the modern world is highly dependent of their use since decades. Despite all the benefits, some pesticides are highly toxic, show environmental persistence or are considered as endocrine disruptors (EDs) [135]. Considerable efforts have been made at national, EU and international level to identify and assess endocrine disruptors and to develop criteria and testing strategies for its identification as a consequence of severe restrictions on substances identified as endocrine disruptors imposed by several pieces of legislation [136]. The European Commission (EC) has a candidate list of 564 substances which are classified as EDs [135,137]). The list includes organochlorine pesticides that were banned by many countries for a long time but are still detected in the environment due their great resistance to photochemical, biological and chemical degradation. Endocrine disrupting activities such as estrogenicity and anti-androgenicity have also been reported for theme [138,139]. Other pesticides were introduced for replacing organochlorine compounds, as organophosphate pesticides (OP) that are commonly used in the present.

Other example is the pyrethroids, due their less persistent and lowers mammalian toxicity. Recent studies have reported that chronic low-level exposure to OP in uterus and in childhood are associated with poorer cognition and behavioral problems [140].

---

*Adapted from: José. L. Vera, Laurens Jans, Ana Isabel Pereira, Virgínia C. Fernandes, C. Mansilha, Valentina. F. Domingues and Cristina Delerue-Matos, Occurrence of pesticides in Portuguese rivers. Analysis by SPME-GC-MS, submitted, 2014.

Due to the very low levels at which these compounds are usually present in the environment (ppb and ppt), and to the complexity of the environmental matrices, preconcentration of the samples previous to analysis is normally required. Nowadays, the technique most widely used for isolation and preconcentration of pesticides from environmental samples is the solid-phase extraction (SPE), owing to some well-known advantages [141]. However SPE implies a method development due to the diversity of choices of solvents and pH conditions, is time required, and presents a high cost per sample.

SPME is thus an alternative extraction method to traditional techniques, allowing complete elimination of solvents, blanks reduction and the extraction is performed in a single step. It is based on the sorption of analytes directly from sample in one-step extraction and pre-concentration of analytes. Other advantage is its ability to allow selective detection of the dissolved analytes in aqueous media containing dissolved organic matter [142,143]. SPME combined with gas chromatography is a method widely used for determination of different group of pesticides in water samples [144].

The Portuguese water quality program, aimed at quantifying the levels of substances responsible for water contamination, was defined in 1983. The more recently legislation on environmental quality standards in the field of water policy is the Directive 2013/39/EC.

In the present study, the development and application of the solid phase microextraction coupled with gas chromatography mass spectrometry (SPME/GC-MS) was selected to achieve high throughput analysis for the determination of pesticides in Portuguese rivers samples. Target compounds included pesticides such as organoclorines, organophosphate, dicarboximide and pyrethroids.

## 3.2 Experimental

### 3.2.1 Site selection and sampling

The selection of sampling sites was primarily focused on areas considered susceptible to be contaminated from human, industrial or agricultural wastewaters. Eighteen samples from twelve rivers from northern Portugal were chosen for this study. The Douro River is one of the longest rivers, sharing its 930 km with Spain and Portugal. Sousa, Cabrum, and Tâmega rivers are tributaries of Douro River. Leça River has its estuary near the port of Leixões. Ria de Aveiro is an important estuarine system on the north-west coast of Portugal. Moscoso and Caima rivers are tributaries from Ria de Aveiro estuarine zone. Minho River at its terminal 70 km constitute the natural border between Portugal and Spain, which includes the main estuarine axis of approximately 40 km. Lima river estuary (NW Portugal) is an urban-industrialized estuary, impacted by input of agricultural runoff and urban and industrial

sewage. Cávado River has its mouth next to the city of Esposende. Ave river basin is located in a very populated region of the country and the water is intensively used for agricultural and industrial purposes. Caima River drains a catchment area, in the north-central region of Portugal. During the sampling, a global position system (GPS) was used to locate the sampling sites Table 3.1. Whenever was possible samples from the two river sides were collected.

Samples were collected in pre-cleaned amber glass bottles (250 mL), previously rinsed several times with the river water, acidified with glacial acetic acid (1%, v/v) were placed in isotherm boxes, and transported to the laboratory immediately after collection, and stored at 4 ºC.

**Table 3.1 Rivers, coordinates, date and sampling points.**

| Nº Sample | River | Date | Coordinates | Sampling point |
|---|---|---|---|---|
| **1** | Douro River | 06-06-2011 | 41º8'36"N; 8º38'53"W | Afurada (near the mouth) |
| **2** | | 06-06-2011 | 41º4'20"N; 8º29'10"W | Lever |
| **3** | Cávado River | 11-07-2010 | 41º30'54"N; 8º46'12"W | Braga |
| **4** | | 11-07-2010 | 41º31´2"N; 8º46´17"W | |
| **5** | Lima River | 13-06-2010 | 41º41´57"N;8º44´36"W | Viana do Castelo |
| **6** | Minho River | 05-06-2010 | 41º56'28"N; 8º44'47"W | Cerveira (Portugal) |
| **7** | | 05-06-2010 | 41º56'27"N; 8º45'2"W | Goian (Spain) |
| **8** | Sousa River | 13-06-2010 | 41º5'26"N; 8º30'39"W | Porto |
| **9** | Tamega River | 21-06-2011 | 41º5'50"N; 8º15'42"W | Amarante |
| **10** | | 27-06-2010 | 41º16'8"N; 8º4'36"W | Amarante |
| **11** | Ria de Aveiro | 16-06-2011 | 40º37'32"N; 8º44'18"W | Aveiro |
| **12** | | 16-06-2011 | 40º36'52"N; 8º44'53"W | Aveiro |
| **13** | Leça River | 07-07-2011 | 41º12´52"N; 8º40´5"W | Leça |
| **14** | | 21-05-2011 | 41º13´5"N; 8º37´27"W | Leça |
| **15** | Cabrum River | 05-08-2011 | 41º5'51"N; 8º2'12"W | Resende |
| **16** | Ribeira Moscoso | 19-06-2011 | 40º49'18"N; 8º24'8"W | Aveiro |
| **17** | Caima River | 27-05-2011 | 40º49'59"N; 8º23'30"W | Vale de Cambra |
| **18** | Ave River | 10-05-2010 | 41º20'57"N; 8º31'13"W | Lousado |

## 3.2.2 Reagents and solutions

Pesticides from different chemical families as hexachlorocyclohexane (isomers α-HCH, β-HCH, γ-HCH, δ-HCH), hexachlorobenzene (HCB), simazine, atrazine, diazinon, vinclozolin, 2,4-dichlorophenoxyacetic acid (2,4-D), alachlor, malathion, aldrin, dieldrin, endrin, isomers (α, β)-endosulfan, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), dichlorodiphenyltrichloroethane (DDT), bifenthrin,

methoxychlor, iprodione and cypermethrin, with purity > 97.0%, were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile HPLC grade were acquired from J. T. Baker (USA was obtained from Merck (Dalmstadt, Germany). Ultrapure water (0.054 µS/cm) was obtained by using a Milli-Q system from Millipore (Milford, MA, USA). The work standard mixture containing the 20 pesticides was prepared by dilution in *n*-hexanethe 100 µg/L concentration and stored at −18 °C. This standard was used both for matrix spike, in order to optimize the extraction conditions (1.0 µg/L) and in the validation study in different concentration levels (0.01 to 3 µg/L). Calibration standards were prepared in the same range of concentrations, by add the work standard mixture in a vial, evaporated with a gentle flow of $N_2$ and finally add 10 mL of the water.

## 3.2.3 Equipments

All weight measurements were done on an analytical balance (Metter Toledo). For pH measurements a GLP 22 pH meter, supplied by Crison, was used. The SPME (100 µm PDMS fiber) procedure was performed with a manual fiber holder assembly supplied by Supelco (USA). A magnetic stirring and heater unit (AGIMATIC-N, Mundilab) for stirring samples, during the SPME process, was used.

Gas Chromatograph Mass Spectrometer equipped with a fused-silica capillary column ZB-XLB (30m x 0.25mm ID, 0.25µm film thickness, Phenomenex) was employed in the separation of pesticides, using helium 99.99% as carrier gas at a 1.3 mL/min flow rate. Injector mode was split/split less injector in the split mode at 260 °C during the chromatographic run. The oven temperature was as followed: initial oven temperature was held at 60 °C for 1 minute, programmed with a gradient of 20 °C/min up to 200 °C where it stays for one minute and then an increase of 5 °C/min up to 245 °C where it stays for 32 minutes. The mass detector conditions were: transfer line temperature 250 °C; ion source temperature 250 °C; ionization mode electron impact at 70 eV.

## 3.2.4 Conditioning and cleaning procedure

Before starting work, the fibers used were conditioned at 250 °C in a current of helium for one hour in the injector of GC-MS, according to factory recommendations (Supelco ®). Routinely, the conditioning was achieved by exposing the fibers to the injector port at 260 °C for 30 minutes prior to use. Several fiber blanks were run to ensure that no interferences from the fibers were present in GC chromatograms. After thermic desorption of the analytes in the GC, the fiber remained in the injector for several minutes to further cleaning at high temperature. The vials and the magnetic stirrers were washed with methanol and n-hexane.

The liner in GC-MS was also replaced and cleaned every week to prevent the accumulation of analytes and thus unwanted peaks in the chromatograms.

### 3.2.5 Extraction Procedure

The sample volume selected for the extraction was 10 mL and the vials were placed on a stir plate. During the extraction step whenever necessary the fiber was stroked to remove air bubbles that appears on the surface of fiber. The SPME was performed during 45 min (extraction time). The pH of the solutions was maintained at 3.5, it was added 2.5% methanol in the 10 mL of water samples and stirring at 700 rpm was kept constant during the study. The vials were immersed in a water bath heated using the magnetic stirring unit. To study the effect of the extraction temperature, the temperature of the heater unit was adapted and was set at 20 ºC, 38 ºC and 60 ºC maintaining constant the remaining operating conditions. A thermometer was used to monitor the water temperature. The samples were analysed using 60ºC of extraction temperature. A 5 µg/L standard solution of the mixture of pesticide was used for this study. Quantification of the analytes was performed by monitoring two transitions, between the precursor ion and the most abundant fragment ion for each compound Table 3.2.

**Table 3.2 Optimized GC-MS acquisition method for 20 pesticides.**

| Pesticides | Rt (min) | Q1>Q2>Q3 |
|---|---|---|
| **α-HCH** | 9.66 | 181, 219,109 |
| **HCB** | 9.82 | 284, 142, 249 |
| **diazinon** | 10.04 | 179, 199, 304 |
| **β-HCH** | 10.36 | 181, 219, 109 |
| **lindane** | 11.04 | 181, 219, 109 |
| **vinclozolin** | 11.38 | 212, 124, 187 |
| **aldrin** | 12.47 | 263, 293, 66 |
| **α-endosulfan** | 14.89 | 241, 195, 209 |
| **p,p´-DDE** | 15.54 | 246, 176, 318 |
| **dieldrin** | 15.68 | 79, 263, 277 |
| **endrin** | 16.35 | 244, 263, 317 |
| **o,p´-DDT** | 16.74 | 235, 165, 81 |
| **p,p´-DDD** | 17.21 | 235, 165, 199 |
| **β-endosulfan** | 17.30 | 195, 335, 339 |
| **bifenthrin** | 19.51 | 165, 166, 141 |
| **metoxichlor** | 20.19 | 227, 152, 165 |
| **iprodione** | 20.32 | 187, 244 |
| **cypermethrin** | 31.72;31.98;32.44 | 163, 181, 91 |
| **fenvalerate** | 38.59;40.63 | 125, 167, 419 |
| **deltamethrin** | 46.62 | 181, 253 |

## 3.3 Results and discussion

### 3.3.1 Chromatographic analysis

The retention times and validation study were done in the FULL mode and also in the SIM (Selected ion monitoring) mode. In SIM mode, the main ion (Q1) was selected and two fragments were also identified (Q2 and Q3) for each analyte.

The results showed that the extraction temperature had influence in SPME performance. Extraction temperature of 60 ℃ favored the extraction of the less volatile compounds, such as aldrin, α-endosulfan, p,p'-DDE, dieldrin, endrin, o,p'-DDT, p,p'-DDD, bifenthrin, methoxychlor, cypermethrin, fenvalerate and deltamethrin, being the selected temperature.

The Table 3.3 shows the limits of detection (LOD) obtained at optimized conditions. The LOD and limit of quantitation (LOQ) were obtained from the ratio signal/noise (S/N) made in the program Xcalibur achieved from the lowest standard of each pesticide.

The very low detection limits of these pesticides (Table 3.3) confer to the present method a very particular interest, allowing reaching the maximal limits for the analysis of pesticides in river waters admitting detection limit equal to three times the background noise.

**Table 3.3 Limited of detection (LOD), Limit of quantification (LOQ) and $R^2$ for each pesticide at 60 ºC of temperature of extraction.**

| Pesticides | LOD (µg/L) | LOQ (µg/L) | $R^2$ |
|---|---|---|---|
| **α-HCH** | 0.0250 | 0.0833 | 0.9912 |
| **HCB** | 0.0025 | 0.0083 | 0.9978 |
| **diazinon** | 0.0031 | 0.0103 | 0.9990 |
| **β-HCH** | 0.0600 | 0.2000 | 0.9991 |
| **lindane (γ-HCH)** | 0.0750 | 0.2500 | 0.9991 |
| **vinclozolin** | 0.0002 | 0.0007 | 0.9994 |
| **aldrin** | 0.0024 | 0.0080 | 0.9983 |
| **α-endosulfan** | 0.0059 | 0.0197 | 0.9991 |
| **p,p´-DDE** | 0.0003 | 0.0010 | 0.9957 |
| **dieldrin** | 0.0007 | 0.0023 | 0.9976 |
| **endrin** | 0.0120 | 0.0400 | 0.9978 |
| **o,p´-DDT** | 0.0018 | 0.0060 | 0.9904 |
| **p,p´-DDD** | 0.0053 | 0.0177 | 0.9935 |
| **β-endosulfan** | 0.0030 | 0.0100 | 0.9977 |
| **bifenthrin** | 0.0070 | 0.0233 | 0.9925 |
| **metoxyclhor** | 0.0016 | 0.0053 | 0.9923 |
| **iprodione** | 0.0273 | 0.0910 | 0.9990 |
| **cypermethrin** | 0.0300 | 0.1000 | 0.9976 |
| **fenvalerate** | 0.0033 | 0.0110 | 0.9917 |
| **deltamethrin** | 0.1500 | 0.5000 | 0.9939 |

## 3.3.2 Application to a rivers analysis

River water samples were collected in four districts (Aveiro, Braga, Porto and Viana do Castelo). Pesticides were divided in three groups (Table 3.4). For the first group the permissible limits are established by environmental quality standards (EQS) determined as maximum allowable concentration (MAC) and annual average (AA) to surface waters according to the directive of the European Union 2008/105/EC and are (α and β) endosulfan,

(α, β, γ and δ) hexachlorocyclohexane, and hexachlorobenzene (HCB). To this group is more appropriate to consider the reference MAC-EQS due to be related to a specific sampling as is our case. In the second group we have the following pesticides: aldrin, dieldrin, endrin, p,p'-DDE, o,p'-DDT, p,p'-DDD also found in the list of the European Union for priority substances and other pollutants. The difference is related with limits established by EQS as an annual average (AA), so when it is refer to this second group will be compared only with the annual average, must take into account further studies for those pesticides that exceeds these limits. In the third group the studied compounds were diazinon, vinclozolin, bifenthrin, metoxychlor, iprodione, cypermethrin, fenvalerate and deltamethrin, not reported in this list of priority substances and other contaminants.

Then it was taken as reference the policy for drinking water according to the Europe where the maximum limit for each pesticide is 0.1 µg/L, considering this an indication for future studies.

**Table 3.4 The adopted maximum limits of the studied pesticides. Adapted from the EU Water Framework Directive 2008/105/CE (for on environmental quality standards (EQS) in the field of water policy) and Directive 98/83/CE (for drinking water)**

| | Pesticides | Inland Surface Waters (AA-EQS - µg/L) | Inland Surface Waters (MAC-EQS - µg/L) |
|---|---|---|---|
| **Group 1** | (α and β)-endosulfan | 0.005 | 0.01 |
| | (α, β, γ and δ) hexachlorocyclohexane | 0.02 | 0.04 |
| | hexachlorobenzene (HCB) | 0.01* | 0.05 |
| **Group 2** | aldrin<br>dieldrin<br>endrin<br>isodrin | Σ = 0.01 | n.a |
| | p,p'-DDT<br>o,p'-DDT<br>p,p'-DDE<br>p,p'-DDD | 0.01 | n.a |
| | DDT total | 0.025 | n.a |
| **Group 3** | diazinon, vinclozolin, bifenthrin, metoxyclhor, iprodione, cypermethrin, fenvalerate and deltamethrin | | Drinking water maximum limit 0.1 µg/L |

n.a = not applicable

The largest number of samples was collected in the Porto district in 2011 Douro River (Afurada) were detected isomers (α and γ) hexachlorocyclohexane at concentrations above the MAC-EQS, as well as (α and β) endosulfan and hexachlorobenzene that do not exceed MAC-EQS, for p,p'-DDE, o,p'-DDT, p,p'-DDD and endrin higher concentrations observed compared with limits for AA-EQS. In Douro River, there were also detected pesticides are cypermethrin as well as vinclozolin with concentration higher than 0.1 µg/L Figure 3.1. In 2011, in Douro River (at Lever) in 2011 was detected γ-hexachlorocyclohexane (commonly called lindane) and α-endosulfan at concentrations above the MAC-EQS. The p,p'-DDE was also detected above that the AA-EQS of DDT total.

Leça River samples (13 and 14) were collected in 2011 and was detected HCB at concentrations below the MAC-EQS and α-endosulfan above the MAC-EQS limit. In sample 14 was also identified endrin. In Sousa River sample was detected HCB, β-HCH, β-endosulfan, aldrin and p, p'-DDE, being the obtained concentrations of HCB, β-HCH, β-endosulfan above MAC-EQS and aldrin, p,p'-DDE above AA-EQS. In sample 9 (2011) was detected HCB, α-endosulfan, vinclozolin, metoxychlor and cypermethrin. The HCB and α-endosulfan concentrations detected were below MAC-EQS. In the case of cypermethrin, the concentration was above the value established for drinking water. In the sample 10 collected in 2010 was detected p,p'-DDE, o, p'-DDT being the sum of these two pesticides greater than AA-EQS of DDT total. The pyrethroid, cypermethrin also was detected above at limit for water consumption.

The Cávado river samples were collected from both margins (sample 3 and 4). In sample 3 was detected isomers (β and γ) hexachlorocyclohexane and HCB at concentrations above the MAC-EQS. p,p'-DDE concentration was higher than the value of the AA-EQS. In sample 4 was identified (α and β) hexachlorocyclohexane, (α and β) endosulfan above the MAC-EQS. Cypermethrin concentration detected in sample 4 was higher than recommended for drinking water. In Ave River sample it was detected vinclozolin, aldrin and α-endosulfan. In Lima river was detected p, p'-DDE at concentrations above the established AA-EQS. Others pesticides were also detected such as bifenthrin, fenvalerate and cypermethrin. The cypermethrin concentration was above of the limit for water consumption. In Minho River was identified β-HCH, p, p'-DDE, β-endosulfan and cypermethrin. The β-endosulfan and p, p'-DDE concentration detected, is above MAC-EQS and AA-EQS respectively. In sample 7 (from Goian) was detected HCB, p, p'-DDD, vinclozolin and cypermethrin.

The sample 11 from Ria de Aveiro was found HCB below the limit for HCB MAC-EQS. Other organochlorine pesticide, endrin, was detected above AA-EQS. The cypermethrin

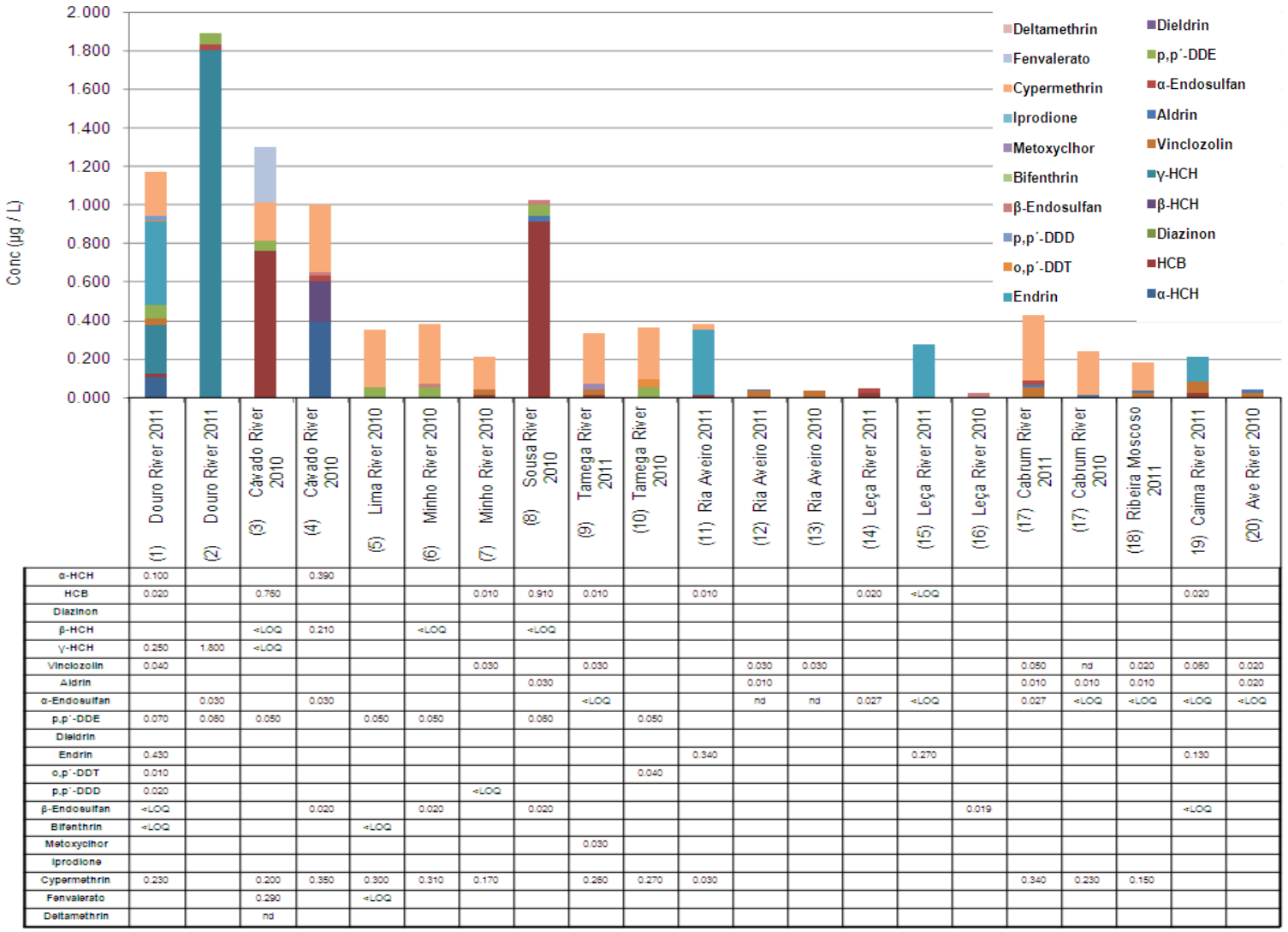
concentration detected was below the limit for drinking water. In sample 12 was detected vinclozolin (below the maximum limit for drinking water) as well as aldrin. In 2010 and 2011, in Cabrum River samples were identified aldrin, α-endosulfan and cypermethrin. In the 2011 was detected vinclozolin too.

In the Ribeira Moscoso sample was detected aldrin, α-endosulfan, vinclozolin and cypermethrin. The cypermethrin concentration found was above of the limits for drinking water.

In Caima River sample was detected endrin, HCB, vinclozolin, (α and β)-endosulfan. The HCB concentration was below the MAC-EQS. The detected concentration for endrin is above limit AA-EQS.

Several authors already described the pesticide detection in Portuguese water samples from rivers. In literature, in studies of sediments of several Portuguese rivers were detected organochlorine ((α and γ) HCH, aldrin, dieldrin, endrin, 4, 4'-DDT, 4, 4'-DDD, 4, 4'-DDE, and endosulfan I) [37]. Other authors also detected (γ-HCH) in the sediments of Douro and Aveiro river and in surface waters [34] and neighborhood soils [145]. The organochlorine pesticides pesticides are very persistent and the problem is that they are lipophilic, with a tendency to bioaccumulate, leading to bioconcentration and biomagnification along the food chain [138]. There are several case-studies reported in literature about the exposure to these pesticides, as for example in southeastern Spain many young men and children were exposed [146]. In Portugal was also reported a similar study that evaluated organochlorine pesticides levels in human serum of the students from Coimbra University [147] and in adipose tissue[52].

Several studies reported pyrethroids in the surface waters from the USA [148] and Spain that reported the presence of cypermethrin in most of several rivers [149].




| | (1) Douro River 2011 | (2) Douro River 2011 | (3) Cávado River 2010 | (4) Cávado River 2010 | (5) Lima River 2010 | (6) Minho River 2010 | (7) Minho River 2010 | (8) Sousa River 2010 | (9) Tamega River 2011 | (10) Tamega River 2010 | (11) Ria Aveiro 2011 | (12) Ria Aveiro 2011 | (13) Ria Aveiro 2010 | (14) Leça River 2011 | (15) Leça River 2011 | (16) Leça River 2010 | (17) Cabrum River 2011 | (17) Cabrum River 2010 | (18) Ribeira Moscoso 2011 | 19) Caima River 2011 | (20) Ave River 2010 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α-HCH | 0.100 | | | 0.390 | | | | | | | | | | | | | | | | | |
| HCB | 0.020 | | 0.760 | | | | 0.010 | 0.910 | 0.010 | | 0.010 | | | 0.020 | <LOQ | | | | | 0.020 | |
| Diazinon | | | | | | | | | | | | | | | | | | | | | |
| β-HCH | | | <LOQ | 0.210 | | <LOQ | | <LOQ | | | | | | | | | | | | | |
| γ-HCH | 0.250 | 1.800 | <LOQ | | | | | | | | | | | | | | | | | | |
| Vinclozolin | 0.040 | | | | | | 0.030 | | 0.030 | | | 0.030 | 0.030 | | | | 0.050 | nd | 0.020 | 0.060 | 0.020 |
| Aldrin | | | | | | | | 0.030 | | | | 0.010 | | | | | 0.010 | 0.010 | 0.010 | | 0.020 |
| α-Endosulfan | | 0.030 | | 0.030 | | | | | <LOQ | | | nd | nd | 0.027 | <LOQ | | 0.027 | <LOQ | <LOQ | <LOQ | <LOQ |
| p,p´-DDE | 0.070 | 0.060 | 0.050 | | 0.050 | 0.050 | | 0.060 | | 0.050 | | | | | | | | | | | |
| Dieldrin | | | | | | | | | | | | | | | | | | | | | |
| Endrin | 0.430 | | | | | | | | | | 0.340 | | | | 0.270 | | | | | 0.130 | |
| o,p´-DDT | 0.010 | | | | | | | | | 0.040 | | | | | | | | | | | |
| p,p´-DDD | 0.020 | | | | | | <LOQ | | | | | | | | | | | | | | |
| β-Endosulfan | <LOQ | | | 0.020 | | 0.020 | | 0.020 | | | | | | | | 0.019 | | | | <LOQ | |
| Bifenthrin | <LOQ | | | | <LOQ | | | | | | | | | | | | | | | | |
| Metoxyclhor | | | | | | | | | 0.030 | | | | | | | | | | | | |
| Iprodione | | | | | | | | | | | | | | | | | | | | | |
| Cypermethrin | 0.230 | | 0.200 | 0.350 | 0.300 | 0.310 | 0.170 | | 0.260 | 0.270 | 0.030 | | | | | | 0.340 | 0.230 | 0.150 | | |
| Fenvalerato | | | 0.290 | | <LOQ | | | | | | | | | | | | | | | | |
| Deltamethrin | | | nd | | | | | | | | | | | | | | | | | | |

**Figure 3.1 Average concentration values expressed in µg/L for pesticides detected at extraction temperature is 60 ºC.**

## 3.4 Conclusion

The DI-SPME-GC/MS method showed good correlation coefficients (R) higher than 0.995 for all the compounds at 60ºC extraction temperature. The samples screening achieved many organochlorine compounds (OC) which use has been subjected to restrictions in the majority of the countries for many years regarding environmental persistence. The other pesticides detected are probably due to the wild use in public health, veterinary and agriculture. The results from the analyzed river samples showed that although some of the investigated pesticides have been banned years ago (organochlorines) they persist in the environment. Among pyrethroids the most often found was cypermethrin.

# CHAPTERS 4

# OCCURRENCE OF PESTICIDES IN RIA DE AVEIRO ESTUARY, PORTUGAL

# CHAPTER 4 Occurrence of pesticides in Ria de Aveiro estuary, Portugal*

## 4.1. Introduction

Protecting the integrity of water source is one of the most essential environmental issues of the 21st century. Research has shown that many compounds can enter the environment, disperse, and persist to a greater extent than first anticipated [150].

Estuaries are important coastal ecosystems. They represent transition systems between freshwater and marine environments and are among some of the most biologically productive areas on Earth. Despite their high productivity, estuaries are ranked amongst the most anthropogenically degraded habitat types on the earth [151]. Human activities jeopardize the functioning of estuaries, and in many cases have caused large scale changes in natural communities [151,152].

Pesticides are defined as substances used to fight pests for the improvement of agricultural production. However, these substances are difficult to degrade and are generally toxic for living organisms. These results in them become long-term toxic agents that frequently accumulate in certain organs of living beings. The properties that make them effective against plagues are precisely the ones that turn them into polluting agents.

The concentration and type of pesticides found in surface waters depend on quite a few different factors. These factors include the season of the year, their solubility in water, their capacity to be retained in the soil, their persistence, the topography of the land, the frequency of rain, and several others [153]. The control of the water in the estuary is important, and sensitive analytical methods capable of detecting traces in the low nanogram-per-liter range are essential for the monitoring of contaminants in the aquatic environment. In addition, high selectivity is required in order to avoid interference by matrix components. To avoid false positives, confirmation techniques using identification points have been implemented or, at the very least, proposed. However, these techniques are often hindered by the low concentrations found in the environment [51].

---

*Adapted from: J. L. Vera, V. F. Domingues, J. M. Costa, C. Delerue-Matos, Occurrence of pesticides in Ria de Aveiro estuary, PORTUGAL, submitted, 2013.

The most commonly used techniques for quantification of organic micro-contaminants in water are analytical methods based on either gas or liquid chromatography, as well as mass or tandem mass spectrometry [51,154]. However, in spite of recent technical progress, the instrumental quantification limits of the micro-contaminants are still high (around µg/L). Therefore, the micro-contaminants quantification in water requires a first step of extraction and pre-concentration in order to detect low levelsThis sample preparation step is, in fact, the critical step of the whole analysis.

The most common method is solid-phase extraction (SPE), which can be used to determine a broad range of contaminants in one analysis. SPE methods are rapid, and offer good recoveries with low detection limits [141,155].

The present work aims to assess the occurrence of a total number of 36 substances (pesticides and degradation products) in waters along Ria de Aveiro estuary in winter and summer.

## 4.2. Materials and methods

The Ria de Aveiro is a shallow coastal lagoon on the northwest coast of Portugal, that consists of a complex network of channels with extensive intertidal zones. The lagoon is connected to the Atlantic Ocean by a narrow channel. Samples were collected at seven sites Table 4.1 in winter (November of 2011) and summer (June of 2012).

**Table 4.1 Places of sampling location data**

| Sample code | Coordinates: latitude/longitude | Area name |
|---|---|---|
| **1** | N40º48'12.40" W08º40'19.84" | Quintas do Norte |
| **2** | N40º41'18.52" W08º43'06.14" | Parque das Cozinhas |
| **3** | N40º39'46.38" W08º43'33.65" | São Jacinto, Molhe |
| **4** | N40º43'43.97" W08º38'59.34" | Bico da Murtosa |
| **5** | N40º41'41.48" W08º36'10.82" | Vilarinho, Cacia |
| **6** | N40º36'31.12" W08º40'55.15" | Ílhavo |
| **7** | N40º40'20.48" W08º27'22.69" | Sernada |

Most of the pesticide sampling points are located in sections of the river that receive run-off waters from various agricultural areas.

Environmental estuary water samples were collected in one liter amber glass bottles and refrigerated at 4 ºC until their subsequent preparation and analysis.

All analytical standards with purity >98% were supplied by Sigma–Aldrich (Steinheim, Germany). *n*-hexane, methanol and ethyl acetate were organic trace analysis grade SupraSolv and were supplied by Merck (Darmstadt, Germany). Acetonitrile was ChromaSolv grade from J.T. Baker (Deventer, Holland). Acetic acid (glacial) 100% was

from Merck (Darmstadt, Germany). Ultra-pure water (0.054 µS/cm) was obtained using a Milli-Q system from Millipore (Milford, MA, USA).

Individual stock standard solutions of 250 mg/L were prepared in methanol (atrazine desethyl, atrazine-D5, atrazine, simazine, vinclozolin, alachlor and malathion), n-hexane (α-HCH, HCB, β-HCH, γ-HCH, δ-HCH, o,p´-DDT, aldrin, α-endosulfan, p,p´-DDE, dieldrin, endrin, p,p´-DDD, β-endosulfan, metoxyclhor bifenthrin, fenpropathrin, λ-cyhalothrin, permethrin, β-cyfluthrin, cypermethrin and fenvalerate) or ethyl acetate (iprodione), with exact and accurate weighting and dilution of the high-purity substances. A mixture was then prepared, also in n-hexane, containing 2 mg/L of each individual compound.

Matrix-standard calibration solutions (residue-free matrix spiked with standards) with concentration levels ranging from 15 to 500 µg/L, were prepared by spiking 500 mL of water just before extraction with different volumes of the 2 mg/L mixture. Stock standard solutions were stored in amber glass-stoppered flasks at 4ºC.

Solid phase extraction was conducted in a SPE vacuum manifold system from Phenomenex (USA). 500 mL of water samples or matrix-standard calibration solutions were spiked with a methanolic solution of deuterated-atrazin standard at 360 µg/L. pH was adjusted at 3 with acetic acid (glacial) and 0.5 % of methanol was added. This was done using LiChrolut EN RP-18 SPE cartridges (100 mg/200 mg, 6 mL) from Merck (Darmstadt, Germany). Extraction procedure with these cartridges has been already described [155]. Briefly, the cartridges were conditioned with ethyl acetate, methanol and water (7 mL : 7 mL : 7 mL) and the elution was achieved with methanol and acetonitrile (2.5 mL : 2.5 mL). After elution, the extracts were evaporated to dryness with a gentle flow of $N_2$. They were re-suspended in 500 µL of n-hexane and analyzed by GC/MS.

Chromatographic analyses were performed on a GC-MS system from the Thermo Electron Corporation. It consisted of a Trace GC Ultra gas chromatograph and a Polaris Q mass spectrometer system, operated in the electron impact ionization (EI) at 70 eV. It was controlled by Xcalibur 1.3 software. Chromatographic separation was performed on a 30 m x 0.25 mm ID ZB-XLB capillary with a film thickness of 0.25 µm column. Oven temperature were programmed as follows: initial temperature 40 ºC (held for 1 min), increased by 30 ºC/min to 220 ºC (held for 5 min), increased again by 10 ºC/min to 290 ºC, and finally was held at this temperature for 10 min. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV with an external ionization source. The inlet temperature was 240 ºC, the carrier gas (high-purity helium-99.9999%) was at 1 mL/min, and the injection volume was 2 µL. The ion source temperature was 250ºC and the electron multiplier was operated at 2100 V.

Quantification analyses were performed in MS mode, but confirmation was performed in the MS/MS mode The parameters: isolation time (IT), the excitation time (ET), the isolation mass window (IMW), excitation voltage (EV) and maximum excitation energy (q) that is defined as the amount of energy that holds a precursor ion in the ion trap during excitation were previous optimized [156] otherwise default value of the equipment was used. The parameters for each pesticide was: alachlor ET-5, IT-24, EV-0.2, IMV-4; aldrin ET-60, EV-2; dieldrin ET-60, EV-2.0, q-0.3, IMV-4; endrin ET-60, EV-2, q-0.3, IMV-1; hexachlorobenzene IT-2, EV-0.2, q-0.45; lindane ET-60; methoxychlor ET-60, EV-2, q-0.3, IMV-1; o,p'-DDT ET-60, IT-36, EV-2, q-0.3; p,p'-DDE and IS ET-5, IT-2, EV-0.2, q-0.45; α-and β endosulfan ET-5, IT-2, EV-0.2, q-0.45; atrazin and atrazine-desethyl ET-5, IT-12, EV-0.2; simazine q-0.2; β-Cyfluthrin IT-2; bifenthrin ET-5, EV-0.2, q-0.3; cypermethrin q-0.45; fenpropathrin ET-5, EV-0.2, q-0.45, IMV-4; fenvalerate and malathion ET-5, IT-12, EV-0.2; λ-cyhalotrhin ET-5, EV-0.2, q-0.45, IMV-4; permethrin IT-2, EV-0.2, q-0.3, IMV-4; deltamethrin ET-5, EV-0.2, IMV-1; iprodione ET-5 and IT-24.

## 4.3. Results and discussion

The validation of the SPE-GC-MS methodology was performed, linear range, matrix effect evaluation and recoveries. Matrix components can affect sample analyses, suppressing or increasing the analytical signal. The results indicating recovery rates exceeding 100% or with a low accuracy are attributed to these effects. These effects depend on not only the characteristics of the compounds but also the actual gas chromatograph conditions, particularly those in the injector, column, and detector [157].

It is explained that matrix protects the analytes from adsorption or degradation during transfer from the injector to the column and thus a maximized amount of analyte reaches the detector, leading to a greater response.

In order to evaluate the linearity of the developed method, six concentration levels of the standard solutions prepared in n-hexane and also in the final extract of the samples were injected in triplicate. The results of the matrix-matched calibration (obtained by plotting the peak areas versus the final concentration of the analytes in the reconstituted extracts) are shown in **Table 4.2**. It also includes the range of concentration tested, as well as the slope and interception and recoveries obtained at 10, 15 and 20 ng/L. As can be seen, determination of the coefficients of determinations were ($R^2$)>0.97 for all compounds.

The limit of detection (LOD) of the method that was defined as the concentration of the pesticides that provided a S/N of 3. LODs in 36 compounds ranged between 0.5 ng/L as for alachlor as pp'-DDE and 26.3 ng/L for cypermethrin, respectively.

A total of 14 samples, were analyzed after the developed procedure. Up to 19 pesticides were detected and properly identified in water from Ria de Aveiro. The results of the analysis can be observed in **Table 4.3**. The herbicides triazines detected were as follows: simazine (summer only) had results lower than 95 ng/L; the degradation product of atrazine (restricted) the atrazine-desethyl (winter only) at 11 ng/L; terbutilazin and terbutilazin-desethyl were analysed (winter only), and achieved values lower than 18 ng/L. The pyrethroids detected were as follows (summer only): bifenthrin was quantified in Parque das Cozinhas and had a result of 102 ng/L (sample 2); λ-cyhalothrin was quantified also (samples 5, 6 and 7); permethrin was quantified in Parque das Cozinhas at 427 ng/L; ß-cyfluthrin was quantified in samples Quintas do Norte, Parque das Cozinhas, São Jacinto and Bico da Murtosa (sampling point 1, 2, 3, and 4); cypermethrin was quantified in Parque das Cozinhas (596 ng/L); fenvalerate was observed in Parque das Cozinhas 665 ng/L, and fenpropathrin in Ilhavo (only winter), which was the only insecticide that achieved 80 ng/L in sample 6. Concerning organochlorines: endrine was lower than LOQ in samples obtained in sampling points 1, 2, 4, 5, 6 and 7 (winter), and in samples 1 and 2 (summer); dieldrin was detected (winter) in samples 5, 6 and 7; o,p'-DDT was quantified (winter) in samples 1, 3, 5 and 7; methoxychlor was quantified (winter) in sample 1. Ureas pesticides were also considered, and linuron was analyzed (winter) and achieved 1.94 µg/L. From the chloroacetanilide family, alaclhor was detected in all samples in winter and summer. S-metolachlor was quantified in sample 1 (winter). The organophosphate dimetoate was analysed (winter) and achieved 15 ng/L (sample 1).

The compounds more frequently quantified in water of Ria de Aveiro were alachor (7 in winter and 2 in summer) and endrin (4 in winter and 2 in summer).

It can be observed that concentrations of pesticides are at a higher level in winter in locations such as Vilarinho, Ílhavo and Sernada. It can also be suggested that during the rainy season pesticides with higher adsorption to soils are occasionally released into the Ria Aveiro. They typically show higher the higher the rainfall, directly relating the presence of the pesticides with runoff processes. However, on the coast side (Quintas do Norte, Parque das Cozinhas, São Jacinto and Bico da Murtosa) the pesticides detected were those usually used by farmers in spring and summer season. Examples of these would be atrazine desethyl and alachlor (high solubility). The observed pesticides levels are in the range of Argentina surface waters [158] and in North Portuguese coast [144] but higher than those obtained in Spanish rivers Spain [159].

To visualize the spatial distributions of data, pesticide concentrations in winter and summer in surface water of the Ria de Aveiro are depicted in Figure 4.1.

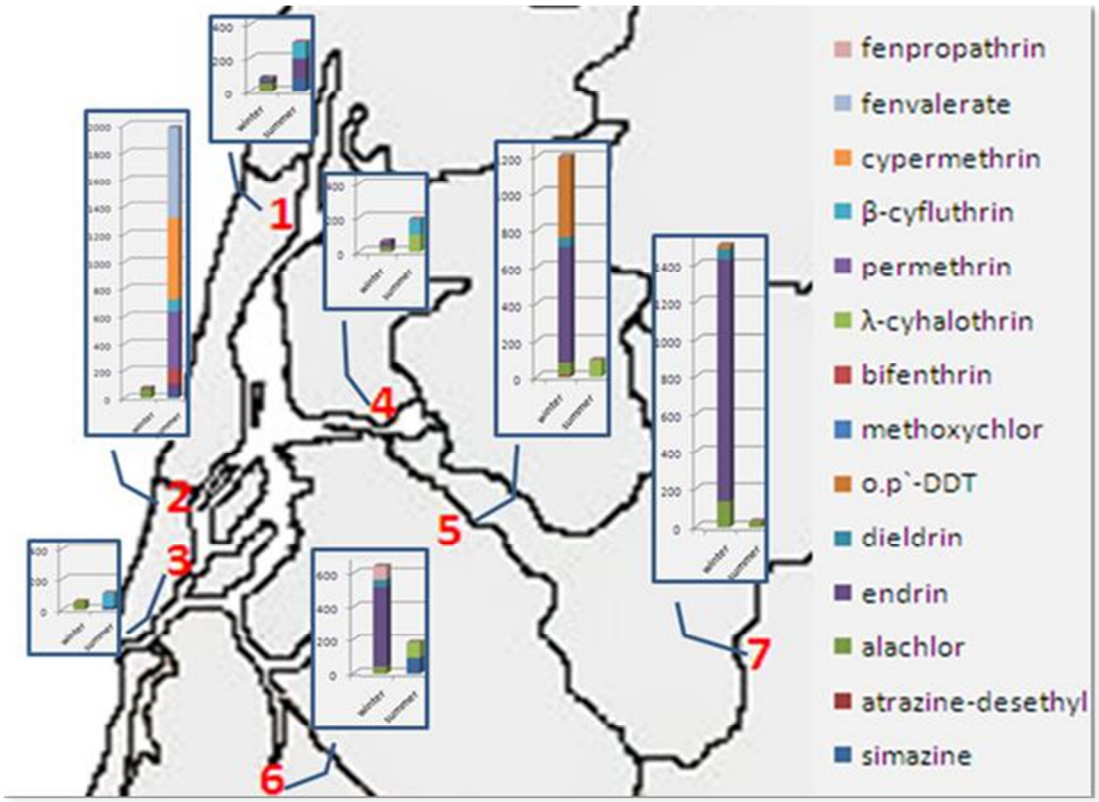



**Figure 4.1 Distribution map of the pesticides concentration (ng $L^{-1}$) in the surface water of the Ria de Aveiro**

**Table 4.2 Method validation data**

| Pesticides | Rt (min) | Linearity (ng/L) | $R^2$ | Calibration curve in matrix | Recoveries (%) (n=3) | | | LOD | LOQ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 100 (ng/L) | 150 (ng/L) | 200 (ng/L) | (ng/L) | (ng/L) |
| α-HCH | 9.33 | 45-500 | 0.992 | Y= -2836 + 68 x | 92±8 | 100±3 | 104±6 | 8.1 | 27 |
| HCB | 9.64 | 15-500 | 0.994 | Y= -80501 + 3722x | 88±1 | 99±2 | 104±4 | 1.1 | 3.7 |
| atrazine desethyl | 14.64 | 30-500 | 0.997 | Y= -884 + 155x | 92±4 | 100±1 | 102±4 | 5.5 | 18.3 |
| atrazine | 9.74 | 15-500 | 0.990 | Y=-22134 + 321x | 88±7 | 100±1 | 105±3 | 3.3 | 11 |
| simazine | 9.74 | 45-500 | 0.967 | Y= -5727 + 130x | 96±2 | 97±2 | 102±3 | 5.4 | 18 |
| β-HCH | 9.90 | 15-500 | 0.990 | Y = -1216 + 60x | 96±7 | 92±5 | 107±8 | 0.8 | 2.7 |
| γ-HCH | 10.65 | 45-500 | 0.992 | Y=-27857 + 950x | 84±9 | 100±5 | 113±9 | 10.8 | 36 |
| vinclozolin | 11.01 | 15-500 | 0.990 | Y=-14168 + 203x | 105±1 | 95±1 | 99±3 | 3.1 | 10.3 |
| alachlor | 11.16 | 15-500 | 0.992 | Y=-21131 + 754x | 90±3 | 93±3 | 102±1 | 0.5 | 1.7 |
| malathion | 11.71 | 15-500 | 0.990 | Y=-14359 + 172x | 102±1 | 100±2 | 98±2 | 4.4 | 14.7 |
| o,p´-DDT | 21.58 | 30-500 | 0.999 | Y= -135 + 65x | 106±1 | 94±1 | 100±1 | 4.8 | 16 |
| aldrin | 11.92 | 15-500 | 0.993 | Y= -41079 +700x | 91±1 | 102±1 | 104±6 | 2.4 | 8.0 |
| α-endosulfan | 14.25 | 15-500 | 0.990 | Y= -6446 + 95x | 103±2 | 98±1 | 99±1 | 3.5 | 11.7 |
| p,p´-DDE | 15.02 | 15-500 | 0.990 | Y=-60484 +1590x | 101±2 | 98±2 | 104±4 | 0.5 | 1.7 |
| dieldrin | 20.77 | 15-500 | 0.999 | Y = -490 + 134x | 96±1 | 95±1 | 100±2 | 4.3 | 14.3 |
| endrin | 21.22 | 56-500 | 0.999 | Y= 782 +24x | 92±1 | 98±1 | 106±5 | 16.9 | 56 |
| p,p´-DDD | 16.60 | 15-500 | 0.991 | Y= -53520 + 1295x | 102±4 | 93±13 | 97±2 | 2.4 | 8.0 |
| β-endosulfan | 16.64 | 45-500 | 0.990 | Y= -10533 +111x | 96±3 | 97±2 | 104±3 | 6.9 | 23 |
| metoxyclhor | 23.40 | 32-500 | 0.996 | Y= 216 + 154x | 101±2 | 102±2 | 100±1 | 9.6 | 32 |
| bifenthrin | 18.75 | 15-500 | 0.992 | Y= -87670 + 2212x | 98±2 | 96±3 | 100±1 | 1.2 | 4.0 |
| fenpropathrin | 19.25 | 45-500 | 0.990 | Y= -16526 + 407x | 101±4 | 96±1 | 100±3 | 5.1 | 17 |
| iprodione | 19.80 | 45-500 | 0.992 | Y= -4334 + 35x | 94±18 | 104±6 | 94±8 | 13.6 | 45 |
| λ-cyhalothrin | 21.51 | 45-500 | 0.990 | Y= -4521 + 113x | 94±1 | 93±6 | 99±2 | 7.5 | 25 |
| permethrin | 24.77 | 100-500 | 0.992 | Y=-35169 + 428x | 105±1 | 96±1 | 100±1 | 19.9 | 66 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| β-cyfluthrin | 28.49 | 100-500 | 0.991 | Y=-39410 + 423x | 95±9 | 97±10 | 99±2 | 20.0 | 67 |
| cypermethrin | 29.67 | 100-500 | 0.993 | Y= -1536 + 62x | 103±3 | 94±9 | 93±6 | 26.3 | 88 |
| fenvalerate | 35.85 | 100-500 | 0.992 | Y=-85453 + 945x | 99±2 | 97±3 | 100±1 | 16.7 | 56 |
| deltamethrin | 43.01 | 100-500 | 0.994 | Y=-21305 + 167x | 95±9 | 91±4 | 98±3 | 24.6 | 82 |
| EPTC | 10.84 | 30-500 | 0.993 | Y= -781 + 143x | 104±10 | 113±9 | 102±8 | 9.1 | 30 |
| 2,4-D Methyl ester | 14.36 | 22-500 | 0.992 | Y= -16+ 11x | 101±9 | 98±11 | 102±10 | 6.5 | 22 |
| Terbuthylazine-desethyl | 14.84 | 30-500 | 0.999 | Y=-336 + 204x | 101±14 | 95±8 | 100±6 | 5.7 | 19 |
| Dimethoate | 15.41 | 30-500 | 0.998 | Y=-1114 + 154x | 100±10 | 97±7 | 101±5 | 2.6 | 8.7 |
| Terbuthylazine | 15.94 | 30-500 | 0.999 | Y=-245 + 207x | 100±8 | 96±6 | 100±5 | 1.6 | 5.3 |
| Linuron | 18.14 | 30-500 | 0.997 | Y=-125 + 184x | 106±10 | 94±9 | 99±10 | 5.5 | 18 |
| S-Metolachlor | 18.30 | 30-500 | 0.997 | Y=-834 + 178x | 117±5 | 94±4 | 103±5 | 4.6 | 15 |
| Pendimethalin | 19.13 | 30-500 | 0.996 | Y=106 +236x | 105±4 | 105±3 | 101±5 | 9.3 | 31 |

## 4.4. Conclusions

The results in this study showed that the developed methodology SPE/GC–MS can be established as a suitable protocol for the simultaneous screening of ultra-trace levels of these pesticides water. Along with the high sensitivity and selectivity inherent to the detector, allows for the identification and quantification of the compounds of interest at concentrations below those reported in national and international legislation. Thirty six pesticides or degradation product in surface water from the Ria de Aveiro were analyzed. The total concentrations ranged from 32 to 3446 ng/L for 19 compounds detected (Σ19 compounds). Some of these pesticides are in fact endocrine disruptors compounds. The result of this research is very meaningful for the protection of environmental and human health.

**Table 4.3 Pesticides detected (ng/L) in the samples between winter of 2011 and summer of 2012**

| Sample code | Season | simazine | atrazine-desethyl | terbuthylazin-desethyl | alachlor | S-metolachlor | endrin | dieldrin | o.p`-DDT | methoxychlor | Bifenthrin | λ-cyhalothrin | Permethrin | β-cyfluthrin | cypermethrin | fenvalerate | fenpropathrin | dimethoate | terbuthylazin | linuron |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | Winter | n.d. | n.d. | <LOQ | 50 | 23 | <LOQ | n.d. | n.d. | n.d | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | 15 | 17 | n.d. |
| | Summer | 78 | n.d. | * | <LOQ | * | 123 | n.d. | n.d. | n.d. | <LOQ | <LOQ | <LOQ | 96 | <LOQ | <LOQ | n.d. | * | * | * |
| **2** | Winter | n.d. | n.d. | n.d. | 66 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 6.9 | n.d. |
| | Summer | 31 | n.d. | * | <LOQ | * | 76 | n.d. | n.d. | n.d. | 102 | <LOQ | 427 | 87 | 596 | 665 | n.d. | * | * | * |
| **3** | Winter | n.d. | n.d. | n.d. | 52 | n.d. | n.d. | n.d. | 1.3 | n.d. | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | Summer | 22 | n.d. | * | <LOQ | * | <LOQ | n.d. | n.d. | n.d. | <LOQ | <LOQ | <LOQ | 88 | <LOQ | <LOQ | n.d. | * | * | * |
| **4** | Winter | n.d. | n.d. | n.d. | 30 | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | Summer | <LOQ | n.d. | * | <LOQ | * | <LOQ | n.d. | n.d. | n.d. | <LOQ | 102 | <LOQ | 89 | <LOQ | <LOQ | n.d. | * | * | * |
| **5** | Winter | n.d. | <LOQ | n.d. | 72 | n.d. | 629 | 50 | 442 | n.d. | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 10 | n.d. |
| | Summer | <LOQ | n.d. | * | <LOQ | * | <LOQ | n.d. | n.d. | n.d. | <LOQ | 98 | <LOQ | <LOQ | <LOQ | <LOQ | n.d. | * | * | * |
| **6** | Winter | n.d. | n.d. | n.d. | 43 | n.d. | 477 | 45 | n.d. | n.d. | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | 80 | n.d. | 7.8 | n.d. |
| | Summer | 95 | n.d. | * | <LOQ | * | <LOQ | n.d. | n.d. | n.d. | <LOQ | 90 | <LOQ | <LOQ | <LOQ | <LOQ | n.d. | * | * | * |
| **7** | Winter | n.d. | n.d. | n.d. | 138 | n.d. | 1295 | 54 | 19 | n.d. | n.d. | <LOQ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1940 |
| | Summer | <LOQ | n.d. | * | 32 | * | <LOQ | n.d. | n.d. | n.d. | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | n.d. | * | * | * |

* These pesticides were not analyzed in summer

# CHAPTERS 5

# SIMULTANEOUS DETERMINATION OF 25 ENDOCRINE DISRUPTING PESTICIDES IN SEDIMENTS FROM RIA DE AVEIRO USING QuEChERS BY GAS CHROMATOGRAPHY-TANDEM, MASS SPECTROMETRY

# CHAPTER 5 Simultaneous determination of 25 endocrine disrupting pesticides in sediments from Ria de Aveiro using QuEChERS by Gas Chromatography-tandem, Mass Spectrometry *

## 5.1. Introduction

There is global phenomenon in regards to the increased use of pesticides in agriculture with many of these pesticides being identified as endocrine disruptors. Endocrine-disrupting pesticides in bottom sediments, with their chronic toxicity, pose a potential threat to aquatic environmental organisms. Sediments act as a pollutant sink and as a carrier and future source of contaminants. These pollutants are not necessarily fixed permanently to sediments, but may be recycled via chemical and biological processes. Behavior of pesticides in sediments is influenced by the nature and properties of pesticides as well as the nature and properties of sediments [160]. Consequently, bottom sediments often become reservoirs of pesticides in the environment. Therefore, the investigation of distribution of endocrine-disrupting pesticides in water and sediment can provide a valuable record of contamination in aquatic environments [161].

The Ria de Aveiro is a lagoon estuary dominated by its connection with the Atlantic Ocean, bringing with it the influence of salt water. The estuary is effected by freshwater from the five main rivers and other small water lines. Runoff can go directly to the Ria, depending on the planning that is done in the territory around the estuary. Ria de Aveiro is surrounded by agricultural areas.

Due to low concentration levels of sediments pollutants, a sample preparation step is needed to determine the type of pollutant involved [162]. To extract contaminants from sediments, a technique strong enough to extract bound residues is necessary [163].

---

*Adapted from: J. L. Vera, V. F. Domingues, A. Almeida, J. M. Costa, C. Mansilha, C. Delerue-Matos, Simultaneous determination of 25 endocrine disrupting pesticides in sediments from Ria de Aveiro using QuEChERS by gas chromatography-tandem, mass spectrometry, submitted, 2014.

The most common methods for extraction are pressurized liquid extraction (PLE) [164], Soxhlet [34,165-167], microwave-assisted extraction (MAE) [168], ultrasound-assisted extracted (UAE) [34,169,170], headspace solid-phase microextraction (HS-SPME) [37], and accelerated solvent extractor (ASE) [171] which can be used to determine a broad range of contaminants in one analysis. These methods are effective, yet time consuming or expensive.

The QuEChERS method has been developed recently for the analysis of non-polar, middle polar and polar pesticides in non-fatty food samples [94]. This method is very flexible, modifiable, and is growing in popularity due to all the benefits described by its name: Quick, Easy, Cheap, Effective, Rugged and Safe. However, its effectiveness is dependent on the analyte properties, matrix composition, equipment, and analytical technique available in the laboratory [172].

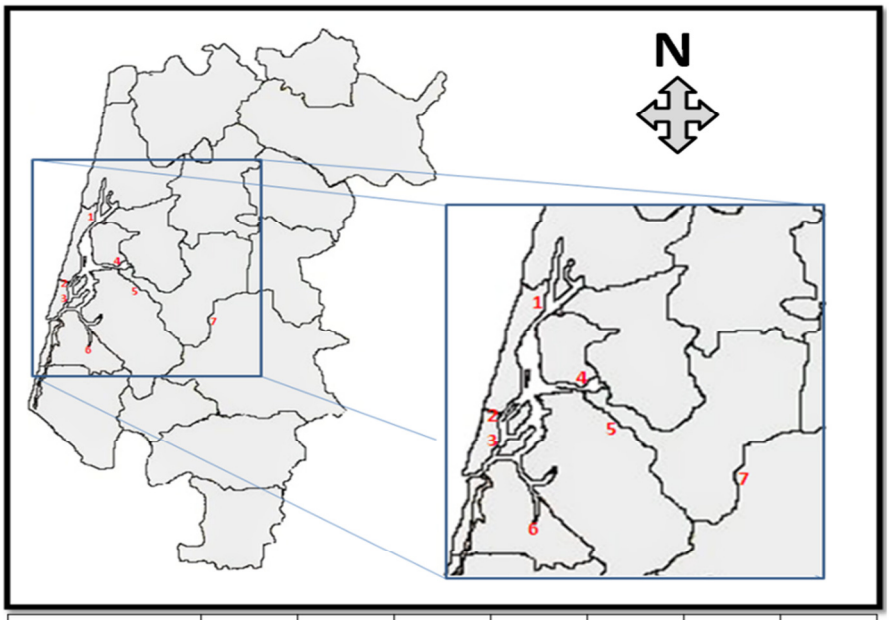
The versatility of QuEChERS has been demonstrated by its acceptance outside of its traditional application areas. This extraction method is unusually applied for the extraction of pesticides from sediments [173-177]. The recovery studies of contaminants from sediments, like soils, can be approach by achieving recoveries in a single sediment or by achieving recoveries for each sediment [91]. However, to perform this evaluation it is essential to have identical sediments without the target pesticide. When it is not possible to compare method performance according to different organic matter content, it is usually performed the evaluation of the recoveries for each sample [163].

For the quantification of the pollutants in sediments, a gas chromatography (GC) coupled with mass spectrometry (MS) detector or Electron capture detector (ECD) as the most commonly used.

The present work aims to assess the occurrence of a total number of 25 substances (pesticides and degradation products) in sediments along Ria de Aveiro estuary, with a sensitive and selective methodology, using QuEChERS extraction and GC-MS.

## 5.2. Materials and methods

Sediment samples were collected in June of 2012 to a depth of 4 cm from seven sampling sites in Ria de Aveiro, and wrapped up in a polyethylene bag. Samples were kept cool during transportation to the laboratory. At the laboratory, the sediments were freeze-dried prior to sample preparation. After drying at 40 ºC and sieving to a grain size of 75 µm samples were stored at room temperature until analysis. The scheme present in Figure 5.1 shows the samples points in the Ria de Aveiro and also the coordinates.




| Sample code | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Coordinates | latitude | N40°48'12.40" | N40°41'18.52" | N40°39'46.38" | N40°43'43.97" | N40°41'41.48" | N40°36'31.12" | N40°40'20.48" |
| | longitude | W08º40'19.84" | W08º43'06.14" | W08º43'33.65" | W08º38'59.34" | W08º36'10.82" | W08º40'55.15" | W08º27'22.69' |

**Figure 5.1 Map showing the point of sampling sample in Ria de Aveiro.**

All analytical standards of pesticides (atrazine desethyl, α-HCH, HCB, atrazine, β-HCH, γ-HCH, vinclozolin, alachlor, malathion, aldrin, α-endosulfan, dieldrin, endrin, p,p´-DDE, o,p´-DDT, p,p´-DDD, β-endosulfan, bifenthrin, fenpropathrin, metoxyclhor, λ-cyhalothrin, permethrin, β-cyfluthrin, cypermethrin, and fenvalerate) with purity >98% were obtained from Sigma-Aldrich Co. (Steinheim, Germany). Methanol, n-hexane and ethyl acetate were of organic trace analysis grade SupraSolv supplied by Merck (Darmstadt, Germany). Acetonitrile (ACN) was ChromaSolv grade from J.T. Baker (Deventer, Holland) and acetic acid (glacial) 100% was acquired from Merck (Darmstadt, Germany).

Individual stock standard solutions containing 250 mg/L were prepared in *n*-hexane by exact weighing of the high-purity substances and accurate dilution. Mixture stock standard solutions containing 2 mg/L of each individual compound, was then prepared, also in *n*-hexane.

QuEChERS commercial products were used for sample preparation. The QuEChERS composition contained in 50 mL teflon centrifugate tube was composed of 6g $MgSO_4$,1.5g NaCl, 1.5g sodium citrate dihydrate, 0.750g of sodium citrate sesquihydrate and the clean up tube contained 150 mg magnesium sulfate, 150 mg PSA, and 50 mg C18. QuEChERS and clean-up were obtained from Unit Chemical Technologies (UCT).

For the initial extraction step, an amount (5 g) of sediments was weighted into a 50-mL centrifuge tube and working standard solution was added: the sample was left for 1 hour at

room temperature to let the *n*-hexane evaporate to ensure that only the analytes (in the fortified sediments) were in contact with the sample. Then 3 mL of water was added and the resulting solution was shaken vigorously for 1 min to prevent salt agglomeration. Then 3.5 mL of ACN were added and shaked. The packet with the QuEChERS content was slowly poured over the sample and maximum speed in vortex device was needed for good homogenization during 1 min. The resulting solution was sonicate for 1 min. After QuEChERS extraction, the extracts were centrifugated during 10 min at 3000 rpm at room temperature. An aliquot of 1.5 mL was sampled from the upper layer and transferred into a 2 mL cleanup tube, vortexed for 1 min and then centrifuged for 5 min at 3000 rpm at room temperature. An aliquot of 1.0 mL from the upper layer was transferred into a vial and evaporated to dryness with a gentle stream of nitrogen. Finally, 250 µL of *n*-hexane was added to dissolve the residue, the resulting solution was shaken vigorously using a vortex device and the extract was then placed into an insert inside the vial.

The GC-MS analysis in this study was performed on a TRACE GC Ultra gas chromatograph Polaris Q coupled with ion trap mass spectrometer (Thermo Fisher Scientific) operated in the electron impact ionization (EI) at 70 eV controlled by Xcalibur 1.3 software. The analytes were separated with a ZB-XLB capillary Column from Phenomenex® (30 m x 0.25 mm ID x 0.25 µm film thickness). Injection (1 µL) was conducted by autosampler (AI3000) in combination with a split/splitless mode and the injector temperature was 240ºC. Ultra high-purity helium was used as carrier gas at 1.3 mL/min (Linde Sogás purity > 99.999 %).

Injector mode was split/split less injector in the split mode at 260 ℃ during the chromatographic run. The oven temperature program was as followed: initial oven temperature was held at 40 ℃ for 1 min, programmed with a gradient of 30 ℃/min up to 220 ℃ where it stays for five minutes and then an increase of 10 ℃/min up to 290 ℃ was performed and maintained during 10 min. The mass detector conditions were: transfer line temperature of 250 ℃ and ion source temperature of 250 ℃.

For the determining of total organic carbon (TOC) in sediments samples a dry oxidative combustion method was performed. A Shimadzu TOC Analyser (model VCSN, Shimadzu $^{(R)}$ Japan) with a solid sample module (SSM-5000A) was used. During the total carbon (TC) analysis, the sample was heated up to 900 ºC in the presence of an oxidation catalyst; the evolved $CO_2$ was carried by synthetic air to the non-dispersive infrared (NDIR) gas analyzer for detection. The NDIR outputs an analog detection signal that forms a peak, and the peak area was measured by the TOC–Control V software. For inorganic carbon (IC) measurement, the sample was acidified with a small amount of orthophosphoric acid (85%) and heated to 200 ºC; the evolved $CO_2$ was detected by NDIR. The calibration curves of TC and IC were generated by analyzing various amounts of D-(+)-glucose anhydrous (from

Scharlau, Sentmenat, Spain) and anhydrous sodium carbonate (from, Nacalai Tesque Inc., Kyoto, Japan), respectively.

## 5.3. Results and Discussion

### 5.3.1 Organic Carbon in sediments

Several studies proved the influence of organic matter in the extraction process, namely QuEChERS extraction and in the efficiency of the analysis. Organic carbon (OC) content was previously determined for the seven collected sediments samples. Using soils as the most similar matrix as sediments it was showed [178] that matrix effect is more pronounced in soils with OC content higher than 2 % for organochlorine pesticide. It was reported in other work [91] the OC influence in the QuEChERS extraction, and the recoveries obtained for soil with high OC were lower. In a study of QuEChERS method applied to soils with different types of characteristics the authors concluded [179] that the pesticides recovery values were highly dependent on the type of soil or OC. The values were below 2 %, in the range between not detected to 1.6 %. Once the best conditions for the studied pesticides analysis were optimized, the validation of the method was carried out. Table 5.1 shows the obtained OC results for the studied sediments. The importance of the determination of OC content for the analytical analysis justified the measurement of this parameter in sediments samples.

**Table 5.1 Values obtained of OC for sediments samples**

| Number of sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| OC (%) | 1.0 | nd. | 0.4 | nd. | 1.6 | 0.7 | 1.0 |

### 5.3.2. Method Validation

The performance of the method, in terms of linearity, recovery, LOD and LOQ, was evaluated. Recoveries were determined by analyzing three blank soil samples spiked at 3 levels (17.5, 35.0 and 52.5 ng/g) concentrations and compared to standard solutions at the same concentration. The results are reported in Table 5.2. The obtained recoveries are between 58.8% (HCB) and 300 % (permethrin) for 16 compounds. Recoveries below 80% and above 120% were also observed, which could be explained by the matrix effect.

Linear response was obtained for all study pesticides with the determination coefficient of ($R^2$) ≥ 0.990. The LOD and LOQ were established using the signal to noise ratio for each compound, a 3:1 ratio was used as the limit of detection while a 10:1 ratio was used as the limit of quantification. The LOD and LOQ ranged, respectively, from 0.3 to 19.4 ng/g and 1.0 to 64.7 ng/g as shown in Table 5.2.

**Table 5.2 Method validation data.**

| Pesticides | Rt (min) | Calibration curve in *n*-hexane | | Matrix calibration curve | | ratio of slopes ($a_{sample}/a_{hexane}$) | LOD (ng/g) | LOQ (ng/g) | Recovery (%)[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $y= b + a_{hexane}x$ | $R^2$ | $y= b + a_{sample}x$ | $R^2$ | | | | 17.5 ng/g | 35.0 ng/g | 52.5 ng/g |
| atrazin desethyl | 7.69 | Y=-15196 +400.1X | 0.991 | Y = -20692+606.9*X | 0.994 | 1.52 | 0.4 | 1.4 | 98 ± 6 | 94 ± 1 | 99 ± 5 |
| α-HCH | 8.11 | Y=-3813 +177.3X | 0.991 | Y = 5514+94.08*X | 0.992 | 0.53 | 4.8 | 14.3 | 168 ± 4 | 173 ± 1 | 175 ± 15 |
| atrazine | 8.21 | Y=-12243 +391.8X | 0.992 | Y = -13686+520.4*X | 0.992 | 1.33 | 1.5 | 5.0 | 92 ± 4 | 113 ± 5 | 113 ± 4 |
| HCB | 8.21 | Y=-15123 +826.6X | 0.999 | Y = -92938+509.8*X | 0.975 | 0.62 | 4.2 | 14 | 95 ± 3 | 105 ± 8 | 129 ± 3 |
| β-HCH | 8.56 | Y= 907+271.7X | 0.994 | Y = -23035+197.6*X | 0.996 | 0.73 | 4.0 | 12.1 | 110 ± 22 | 119 ± 14 | 168 ± 14 |
| Lindane | 9.02 | Y=-803 + 151.9X | 0.998 | Y = -6499+174.4*X | 0.993 | 1.15 | 2.8 | 8.5 | 90 ± 3 | 113 ± 2 | 140 ± 1 |
| vinclozolin | 9.26 | Y=-6565+275.6X | 0.995 | Y = -11732+426.5*X | 0.990 | 1.55 | 1.2 | 4.0 | 143 ± 6 | 84 ± 1 | 88 ± 6 |
| alachlor | 9.37 | Y=-17153+583.6X | 0.992 | Y = -23364+925.7*X | 0.991 | 1.59 | 0.7 | 2.4 | 103 ± 1 | 81 ± 12 | 92 ± 1 |
| malathion | 9.85 | Y=-4597+171.4X | 0.996 | Y = -13422+330.0*X | 0.992 | 1.93 | 0.2 | 0.8 | 106 ± 1 | 81 ± 12 | 79 ± 3 |
| aldrin | 10.32 | Y=-31540+1053.0X | 0.994 | Y = -70983+928.3*X | 0.991 | 0.88 | 0.3 | 1.0 | 127 ± 5 | 132 ± 3 | 134 ± 8 |
| α-endosulfan | 12.86 | Y=-25542+763.9X | 0.995 | Y = 23179.7+925.9*X | 0.998 | 1.21 | 1.3 | 4.3 | 117 ± 4 | 127 ± 2 | 129 ± 6 |
| dieldrin | 13.66 | Y=-9882+419.8X | 0.990 | Y = -14254+427.0*X | 0.991 | 1.02 | 3.6 | 15.9 | 106 ± 4 | 91 ± 2 | 104 ± 7 |
| endrin | 13.67 | Y=-19965+331.7X | 0.990 | Y = -19648+409.3*X | 0.997 | 1.23 | 1.7 | 5.6 | 297 ± 7 | 218 ± 2 | 246 ± 7 |
| pp'-DDE | 14.29 | Y=-85220+2578.4X | 0.997 | Y = -152148+3205.7*X | 0.996 | 1.24 | 0.4 | 1.3 | 58 ± 1 | 46 ±1 | 49 ± 1 |
| o,p'-DDT | 14.59 | Y=-305977+10345.6X | 0.995 | Y = -895806+11881.5*X | 0.996 | 1.15 | 1.0 | 3.2 | 94 ± 19 | 38 ± 4 | 60 ± 3 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DDD | 14.96 | Y=-6281+215.0X | 0.994 | Y = -7817+304.3*X | 0.991 | 1.42 | 3.5 | 11.7 | 108 ± 4 | 84 ± 1 | 84 ± 4 |
| β-endosulfan | 15.09 | Y=-6319+154.8X | 0.991 | Y = -5230+183.8*X | 0.995 | 1.19 | 2.7 | 8.9 | 76 ± 2 | 76 ± 1 | 80 ± 4 |
| bifentrhin | 16.59 | Y=-86954+2507.2X | 0.994 | Y = -154405+4868.4*X | 0.995 | 1.94 | 0.4 | 1.3 | 81 ± 1 | 106 ± 2 | 111 ± 1 |
| fenpropatrhin | 16.92 | Y=-30627+360.3X | 0.991 | Y = -54523+1100.0*X | 0.995 | 3.05 | 1.2 | 4.0 | 79 ± 2 | 96 ± 3 | 98 ± 1 |
| methoxyclhor | 16.99 | Y=-55316+1617.4X | 0.992 | Y = -312041+1856.2*X | 0.998 | 1.15 | 1.4 | 4.7 | 115 ± 3 | 86 ± 1 | 117 ± 2 |
| λ-cyhalothrin | 17.97 | Y=-17822+603.5X | 0.993 | Y = -73561+1416.8*X | 0.994 | 2.35 | 0.8 | 2.6 | 51 ± 3 | 46 ± 1 | 58 ± 3 |
| permethrin | 19.07 | Y=-125285+2790.1X | 0.990 | Y = -198295+5075.6*X | 0.996 | 1.82 | 0.5 | 1.8 | 165 ± 2 | 148 ± 26 | 158 ± 1 |
| β-cyfluthrin | 20.01 | Y=-13436+534.3X | 0.991 | Y = -80422+1396.5*X | 0.994 | 2.61 | 3.2 | 10.5 | 102 ± 10 | 61 ± 11 | 61 ± 3 |
| cypermethrin | 20.39 | Y -15004+422.7X | 0.990 | Y = -43371+956.7*X | 0.994 | 2.26 | 3.1 | 10.2 | 126 ± 8 | 132 ± 5 | 131 ± 3 |
| fenvalerate | 21.48 | Y=-18044+651.2X | 0.992 | Y = -16440+1867.4*X | 0.925 | 2.87 | 1.4 | 4.6 | 49 ± 3 | 79 ± 1 | 82 ± 2 |

Rt, retention time, y, peak area; a, slope; x, concentration; b, intercept.

[a] Mean percent recovery ( RSD of pesticides in sediments at 17.5, 35.0, and 52.5 ng/g fortification levels (n = 3).

The regression equations obtained in the calibration matrix extract with that obtained in the solvent (n-hexane) were compared. Thus, a matrix-matched calibration curve (linearity range of 17.5-175 ng/g), with a mean OC content, was performed. The ratio value of 0.8–1.2 was established as acceptable [113]. As a result, and as can be seen in Table 5.2, some pesticides show an important matrix effect in the sediments with respect to the standards. We observed matrix effect atrazine desethyl, α-HCH, atrazine, HCB, β-HCH, vinclozolin, alachlor, malathion, DDD, bifentrhrin, fenpropatrhin, λ-cyhalotrhin, permetrhin, β-cyfluthrin, cypermethrin, and fenvalerate. No matrix effect was observed for the other compounds. Thus, to compensate this matrix effect and to avoid any overestimation, a matrix-matched calibration, was used.

### 5.3.3. Sediments samples analysis

To further demonstrate the applicability of the proposal method, for the monitoring of the selected pesticides residues in sediments, 7 samples were analysed. Table 5.3 shows the obtained results. Atrazine desethyl (degradation product of atrazine) was detected in 71.4 % of the samples, the pesticide β-HCH was detected in 28.6 % of the samples and the pesticide fenvalerate was detected in all samples. These results are consistent with other study that state that triazines and pyrethroids are commonly used in Aveiro district [180]. Some organochlorine pesticide were also detected due to the fact of being very persistent in the environment, such β-HCH which was also reported in carrot samples collected in the fields near to Ria de Aveiro [91].

The levels found for atrazine desethyl were lower than 5.5 ng/g, for β-HCH were lower than the LOQ, and for fenvalerate were between 6.7 to 27.2 ng/g.

**Table 5.3 Concentration of pesticides detected in the sediment from Ria de Aveiro.**

| Pesticides | Concentration ng $g^{-1}$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | Sample 7 | Number of positive sample | % Frequency |
| atrazine desethyl | 5.5 | 4.7 | nd | 4.0 | nd | 5.0 | <LOQ | 5 | 71.4 |
| β-HCH | nd | <LOQ | nd | <LOQ | nd | nd | nd | 2 | 28.6 |
| fenvalerate | 23.4 | 19.7 | 6.7 | 17.8 | 14.8 | 27.2 | 7.6 | 7 | 100 |

nd- not detected

Representative chromatograms of a sediment sample are presented in Figure 5.2 n with the detection of atrazine desethyl and in Figure 5.3 with the detection of fenvalerate in sample 4.

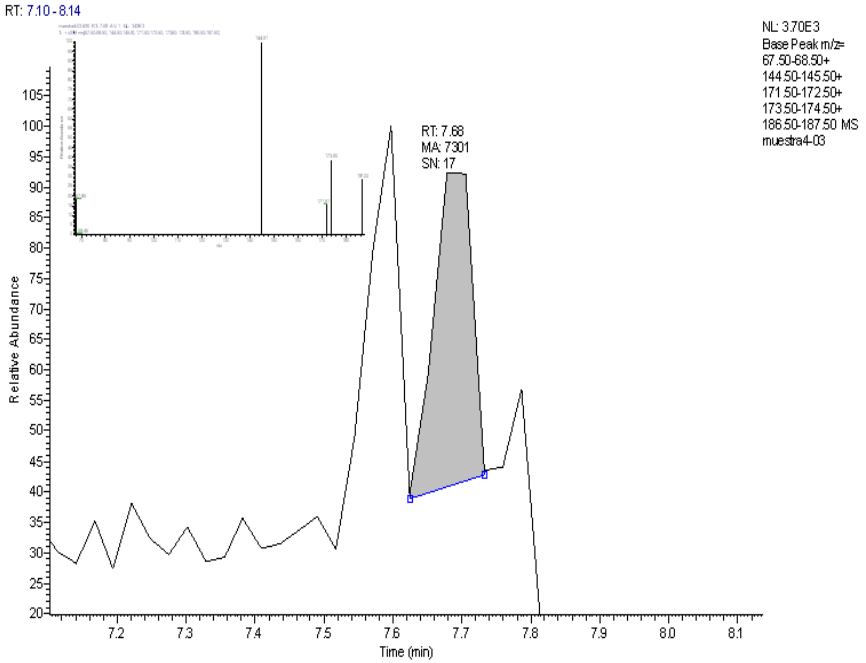



**Figure 5.2 Chromatogram and spectra of atrazine desetyl detected in sample 2**

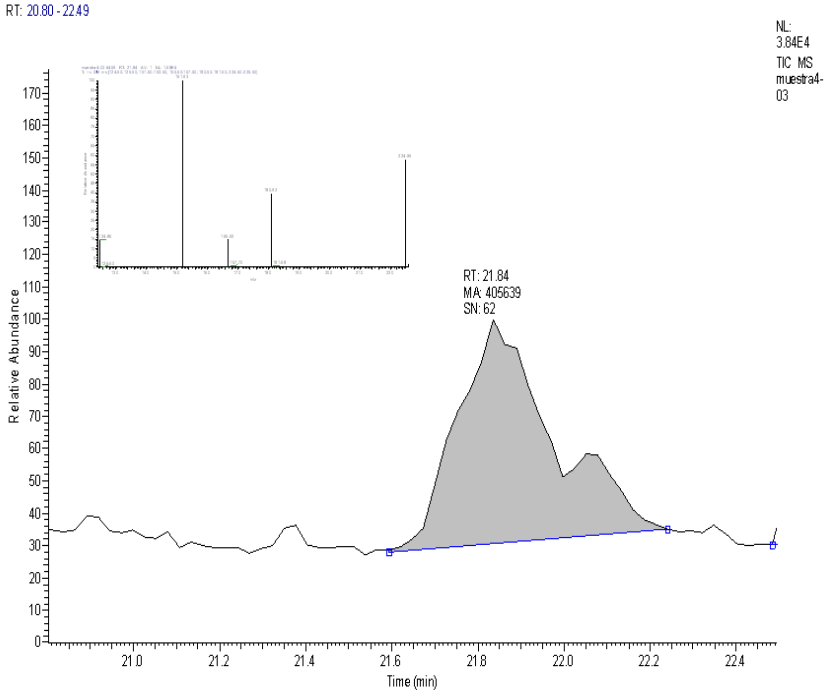



**Figure 5.3 Chromatogram and spectra of fenvalerate detected in sample 4.**

In literature there are some studies reporting the presence of atrazine desethyl, β-HCH, and fenvalerate in sediment samples from different places in the world. Atrazine desethyl was detected in sediments from France [181,182], Canada [183], and Greece [184]. The highest [182] with a concentration of 22370 ng/g and the lowest level [181] with a 1.5 ng/g in sediments were reported in France.

HCH was quantified in sediments from China [161,185], Egypt [171], and Spain [170] with the highest value [170] (26 ng/g). The presence of α-HCH was also achieved in Portuguese coastal areas with levels between 1.0 and 3.5 ng/g [37].

In the case of fenvalerate, studies from China [161,185] and from USA [186] reported its presence. The fenvalerate level found in this study is higher than the levels found in these two countries. China [185] achieved the lowest fenvalerate level.

**Table 5.4 Pesticide levels (ng/g) found in sediments in different countries.**

| Sampling Site | Concentration (ng $g^{-1}$) | | | Ref. |
|---|---|---|---|---|
| | atrazine desethyl | β-HCH | fenvalerate | |
| Portugal (Ria Aveiro) | nd-5.5 | nd-<LOQ | 6.7-27.7 | Present Study |
| China | | 1.5–6.0 | 0.045–0.158 | [161] |
| China | | 2.20-5.68 | nd-0.047 | [185] |
| Egypt | | nd-3.5* | | [171] |
| Spain | | nd-26 | | [170] |
| USA | | | <0.184-<0.114 | [186] |
| France | 1.5 | | | [181] |
| France | nd-22370 | | | [182] |
| Canada | <10-100 | | | [183] |
| Greece | 235 | | | [184] |

nd-not detected

*the authors express the concentration of HCH as the sum of α, β, γ, and δ.

.

## 5.4. Conclusion

A simple, fast, and sensitive method has been developed for the determination of 25 multiclass pesticides in sediment samples. The method is based on the extraction of the pesticides using QuEChERS extraction and direct analysis of the extracts by GC-MS/MS. The analytical method was developed and validated, showing good linearity, with determination coefficients ($R^2$) higher than 0.995 for all compounds. The quantification was carried out using a matrix matched calibration to minimize the existence of the matrix effect. The ranges of the LOD and LOQ in the sediments were 0.3 to 19.4 ng/g and 1.0 to 64.7 ng/g, respectively.

Results showed the presence of atrazine desethyl, β-HCH, and fenvalerate in several samples, with concentrations ranging until 5.5 ng/g, <LOQ, and 27.2 ng/g respectively. The obtained results in sediments samples collected in Ria de Aveiro are related to the persistent in the environmental for organochlorine pesticides, which was also reported in the literature in other matrix samples. Regarding to triazines and pyrethoids pesticides were detected due to the application of this two families in the intensive agriculture to the field in Aveiro region. In literature there are few work reporting the detection of pesticides in sediments applying QuEChERS methodology.

# CHAPTERS 6

# Conclusions and Prospects

# CHAPTER 6 Conclusions and prospects

## 6.1 Concluding remarks

The increase in demand for agro-products, coupled with changing regional climates, has caused a rise in consumption and application rates of pesticides. Frequent use of these pesticides, along with a lack of timely degradation, has caused a persistence of these chemicals in the environment.

The knowledge obtained during this study aimed to contribute to the evaluation of EDP in the environment.

The optimization of the GC-MS/MS system requires the assessment of the influence of IT-MS parameters. This was accomplished by using the changing one-factor-at-a-time (OFAT) approach, which was important for an adequate quantification and identification of the studied EDP. Multiple linear regressions were used as an important tool to predict the "best" combination of IT-MS parameters. This resulted in maximizing the analytical response, with the optimum values for most of the pesticides studied being as follows: q=0.45, IT=12, EV=0.2, IMW= 4, and ET=5 of the MS/MS system.

EDP was detected in Portuguese rivers in the northern region; 910 ng/L atrazin desethyl, 390 ng/L α-HCH, 760 ng/L HCB, 210 ng/L β-HCH, 1800 ng/L lindane, 60 ng/L vinclozolin, 30 ng/L aldrin, 30 ng/L α-endosulfan, 430 ng/L endrin, 70 ng/L pp'-DDE, 40 ng/L o,p'-DDT, 20 ng/L DDD, 20 ng/L β-endosulfan, 10 ng/L bifenthrin, 30 ng/L methoxyclhor, 310 ng/L λ-cyhalothrin, 350 ng/L cypermethrin, 290 ng/L fenvalerate, and 100 ng/L deltamethrin. The rivers with the most pesticides detected were the River Cávado and the River Douro. The River Cávado, located in an urbanized environment, is disturbed by human activities. The Douro River crosses fields with extensive agricultural activity, which explained the contamination over the years.

Two type of samples were collected on the Ria de Aveiro estuary: surfaces water and sediments. Two water samples were collected in the summer of 2011 and the winter of 2012. The maximum quantification of the EDP achieved was 11 ng/L atrazin desethyl, 95 ng/L simazine , 18 ng/L terbuthylazin desethyl, 1940 ng/L terbuthylazin, 138 ng/L alachlor, 23 ng/L s-metolachlor, 4 ng/L metolachlor, 54 ng/L dieldrin, 1295 ng/L endrin, 442 ng/L o,p'-DDT, 102 ng/L bifentrhin, 80 ng/L fenpropathrin, 102 ng/L λ-cyhalothrin, 427 ng/L permethrin, 96 ng/L β-cyfluthrin, 596 ng/L cypermethrin, 665 ng/L fenvalerate, 15 ng/L dimethoate, and 1940 ng/L linuron. A sediment sampling was performed and the pesticides quantified were 5 ng/g atrazine desethyl and 28 ng/g fenvalerate. These two pesticides detected in the sediment samples were also found in the water samples.

## 6.2 Future Perspectives

Recent advances, particularly in sample preparation and analytical euipment, allow for the development of faster, sensitive, precise, accurate, and greener analytical methodologies. Consequently, in the future it should be easier to extend the monitoring of EDPs in the environment. This will assist in the better understanding of their fate in regards to (bio) degradation, environmental compartments partition, and ecotoxicological effects.

With the knowledge acquired it would be desirable to use the methodologies in assessing the quality of the environment in Paraguay.

The establishment of partnerships with the Grupo de Reação e Análises Químicas will maintain a fruitful collaboration, with assurances of a continuous update.

# Bibliography


(1) European Commission, *2012*,http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm#priority_list.

(2) Sharpe, R. M.; Irvine, D. S. *BMJ (Clinical research ed.)* **2004**, *328*, 447-451.

(3) Diamanti-Kandarakis, E.; Bourguignon, J.-P.; Giudice, L. C.; Hauser, R.; Prins, G. S.; Soto, A. M.; Zoeller, R. T.; Gore, A. C. *Endocrine Reviews* **2009**, *30*, 293-342.

(4) Matisová, E.; Hrouzková, S. *International Journal of Environmental Research and Public Health* **2012**, *9*, 3166-3196.

(5) Louekari, T. A. a. K. *Research for management of environmental risks from endocrine disrupters– contexts, knowledge base, methodologies and strategies*; Finnish Environment Institute (FEI), 2001.

(6) McLachlan, J. A. *Endocrine Reviews* **2001**, *22*, 319-341.

(7) LaFleur, A. D.; Schug, K. A. *Analytica Chimica Acta* **2011**, *696*, 6-26.

(8) Hernández, A. F. P., Tesifón Tsatsakis, Aristidis M. Requena, Mar Alarcón, Raquel López-Guarnido, Olga *Toxicology* **2013**.

(9) Mezcua, M.; Martínez-Uroz, M. A.; Gómez-Ramos, M. M.; Gómez, M. J.; Navas, J. M.; Fernández-Alba, A. R. *Talanta* **2012**, *100*, 90-106.

(10) Crisp, T. M.; Clegg, E. D.; Cooper, R. L.; Wood, W. P.; Anderson, D. G.; Baetcke, K. P.; Hoffmann, J. L.; Morrow, M. S.; Rodier, D. J.; Schaeffer, J. E.; Touart, L. W.; Zeeman, M. G.; Patel, Y. M. *Environmental health perspectives* **1998**, *106 Suppl 1*, 11-56.

(11) Foster, W. G.; Agzarian, J. *Journal of Toxicology and Environmental Health, Part B* **2008**, *11*, 152-161.

(12) European Commission, *2014*,http://ec.europa.eu/environment/archives/docum/99706sm.htm.

(13) Ch. Groshart, P. C. O. "EUROPEAN COMMISSION DG ENV Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption- preparation of a candidate list of substances as a basis for priority setting. Final report (incorporating corrigenda to final report dated 21 June 2000)," 2000.

(14) Geyer, H.; Rimkus, G.; Scheunert, I.; Kaune, A.; Schramm, K.-W.; Kettrup, A.; Zeeman, M.; Muir, D. G.; Hansen, L.; Mackay, D. In *Bioaccumulation – New Aspects and Developments*; Beek, B., Ed.; Springer Berlin Heidelberg, 2000; Vol. 2J; pp 1-166.

(15) EPA, *2013*,http://www.epa.gov/pesticides/about/index.htm#what_pesticide.

(16) European Crop Protection *2013*,http://www.ecpa.eu/content/about-ecpa.

(17) Svetlana Hrouzková, E. M. In *Pesticides- Advances in Chemical and Botanical Pesticides*; InTech: Croatia, 2012; pp 99-126.

(18) Zhang, Z.; Gong, Y.; Shan, W.; Jian, Q.; Shen, Y.; Liu, X. *Se pu = Chinese journal of chromatography / Zhongguo hua xue hui* **2012**, *30*, 91-4.

(19) Arthur G. Hornsby, R. D. W. a. A. E. H. *Pesticide Properties in the Enviroment*; Springer: New York, 1996.

(20) Mnif, W.; Hassine, A. I. H.; Bouaziz, A.; Bartegi, A.; Thomas, O.; Roig, B. *International Journal of Environmental Research and Public Health* **2011**, *8*, 2265-2303.

(21) EU Reference Laboratories for Residues of Pesticides, *2013*,http://www.crl-pesticides-datapool.eu/default.aspx.

(22) Cocco, P. *Cadernos de Saúde Pública* **2002**, *18*, 379-402.

(23) McKinlay, R.; Plant, J. A.; Bell, J. N. B.; Voulvoulis, N. *Environment International* **2008**, *34*, 168-183.

(24) National Pesticide Information Center (NPIC), *2013*,http://npic.orst.edu/.

(25) Liu, L.; Hu, J.-x.; Wang, H.; Chen, B.-j.; He, Z.; Xu, L.-c. *Environmental Toxicology and Pharmacology* **2010**, *30*, 251-256.

(26) Zhang, J.; Zhu, W.; Zheng, Y.; Yang, J.; Zhu, X. *Reproductive Toxicology* **2008**, *25*, 491-496.

(27) Naidoo, V.; Katsu, Y.; Iguchi, T. *General and Comparative Endocrinology* **2008**, *159*, 188-195.

(28) Fox, J. E. *Environmental Health Perspectives* **2004**, *112*.

(29) Mori, N. M. T. *Pyrethroids from Chrysanthemum to Modern Industrial Insecticide* 2011.

(30) Mikamo, E.; Harada, S.; Nishikawa, J.-i.; Nishihara, T. *Toxicology and Applied Pharmacology* **2003**, *193*, 66-72.

(31) D Maycock, M. C., C Atkinson and I Johnson "Proposed EQS for Water Framework Directive Annex VIII substances: Pendimethalin (For consultation) ", 2012.

(32) Ruth Bevan, P. H., Louise Youngs, Mick Whelan, Emma Goslan, Jitka macadam, Phil Holmes and Tom Persich "A review of latest endocrine disrupting chemicals research implications for drinking water," The Institute of Environment and Health (IEH), 2012.

(33) Diário da República, Decreto-Lei n.º 306/2007, *2013*,http://dre.pt/pdf1s/2007/08/16400/0574705765.pdf.

(34) Villaverde, J.; Hildebrandt, A.; Martínez, E.; Lacorte, S.; Morillo, E.; Maqueda, C.; Viana, P.; Barceló, D. *Science of The Total Environment* **2008**, *390*, 507-513.

(35) Lino, C. M.; Silveira, M. I. N. d. *Environmental Research* **2006**, *102*, 339-351.

(36) Cerejeira, M. J.; Viana, P.; Batista, S.; Pereira, T.; Silva, E.; Valério, M. J.; Silva, A.; Ferreira, M.; Silva-Fernandes, A. M. *Water Research* **2003**, *37*, 1055-1063.

(37) Carvalho, P. N.; Rodrigues, P. N. R.; Basto, M. C. P.; Vasconcelos, M. T. S. D. *Chemosphere* **2009**, *75*, 595-600.

(38) Palma, P.; Kuster, M.; Alvarenga, P.; Palma, V. L.; Fernandes, R. M.; Soares, A. M. V. M.; López de Alda, M. J.; Barceló, D.; Barbosa, I. R. *Environment International* **2009**, *35*, 545-551.

(39) de Almeida Azevedo, D.; Lacorte, S. l.; Vinhas, T.; Viana, P.; Barceló, D. *Journal of Chromatography A* **2000**, *879*, 13-26.

(40) INE, I. P.; Instituto Nacional de Estatística, I. P., Ed.; Statistics Portugal: Lisboa-Portugal, 2009.

(41) Ferreira, A. M.; Cortesão, C.; Castro, O. G.; Vale, C. *Science of The Total Environment* **1990**, *97–98*, 627-639.

(42) Batista, S.; Silva, E.; Galhardo, S.; Viana, P.; Cerejeira, M. J. *International Journal of Environmental Analytical Chemistry* **2002**, *82*, 601-609.

(43) Blasco, C.; Lino, C. M.; Picó, Y.; Pena, A.; Font, G.; Silveira, M. I. N. *Journal of Chromatography A* **2004**, *1049*, 155-160.

(44) Teixeira, M. J.; Aguiar, A.; Afonso, C. M. M.; Alves, A.; Bastos, M. M. S. M. *Analytica Chimica Acta* **2004**, *513*, 333-340.

(45) Antunes, P.; Gil, O. *Chemosphere* **2004**, *54*, 1503-1507.

(46) Gonçalves, C.; Alpendurada, M. F. *Talanta* **2005**, *65*, 1179-1189.

(47) Campos, A.; Lino, C. M.; Cardoso, S. M.; Silveira, M. I. N. *Food Additives & Contaminants* **2005**, *22*, 642-646.

(48) Pérez, J.; Loureiro, S.; Menezes, S.; Palma, P.; Fernandes, R.; Barbosa, I.; Soares, A. *Environmental Science and Pollution Research* **2010**, *17*, 688-702.

(49) Gonçalves, C. M.; Esteves da Silva, J. C. G.; Alpendurada, M. F. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 6227-6235.

(50) Fernandes, V. C.; Domingues, V. F.; Mateus, N.; Delerue-Matos, C. *Journal of Agricultural and Food Chemistry* **2011**, *59*, 7582-7591.

(51) Mansilha, C.; Melo, A.; Ferreira, I.; Pinho, O.; Domingues, V.; Pinho, C.; Gameiro, P. *Bulletin of Environmental Contamination and Toxicology* **2011**, *87*, 312-318.

(52) Fernandes, V. C.; Pestana, D.; Monteiro, R.; Faria, G.; Meireles, M.; Correia-Sá, L.; Teixeira, D.; Faria, A.; Calhau, C.; Domingues, V. F.; Delerue-Matos, C. *Biomedical Chromatography* **2012**, *26*, 1494-1501.

(53) Silva, E.; Pereira, A. C.; Estalagem, S. P.; Moreira-Santos, M.; Ribeiro, R.; Cerejeira, M. J. *Journal of Environmental Quality* **2012**, *41*, 1413-1426.

(54) Silva, E.; Mendes, M. P.; Ribeiro, L.; Cerejeira, M. J. *Environmental Science and Pollution Research* **2012**, *19*, 2667-2680.

(55) Caetano, A. L.; Goncalves, F.; Sousa, J. P.; Cachada, A.; Pereira, E.; Duarte, A. C.; da Silva, E. F.; Pereira, R. *Journal of Environmental Monitoring* **2012**, *14*, 925-936.

(56) Molinova, B.; Pertile, E.; Brezinova, M. *Carpathian Journal of Earth and Environmental Sciences* **2013**, *8*, 133-142.

(57) Correia-Sá, L.; Fernandes, V.; Calhau, C.; Domingues, V.; Delerue-Matos, C. *Food Anal. Methods* **2013**, *6*, 587-597.

(58) Zhang, C. *Fundamentals of Environmental Sampling and Analysis*, 2007.

(59) García Pinto, C.; Herrero Martín, S.; Pérez Pavón, J. L.; Moreno Cordero, B. *Analytica Chimica Acta* **2011**, *689*, 129-136.

(60) Albaseer, S. S.; Nageswara Rao, R.; Swamy, Y. V.; Mukkanti, K. *Journal of Chromatography A* **2010**, *1217*, 5537-5554.

(61) Bedendo, G. C.; Jardim, I. C. S. F.; Carasek, E. *Talanta* **2012**, *88*, 573-580.

(62) John M. Kokosa, A. P., Michael A. Jeannot *Solvent Microextraction, Theory and Practice*, 2009.

(63) Naeeni, M. H.; Yamini, Y.; Rezaee, M. *The Journal of Supercritical Fluids* **2011**, *57*, 219-226.

(64) Zacharis, C. K.; Rotsias, I.; Zachariadis, P. G.; Zotos, A. *Food Chemistry* **2012**, *134*, 1665-1672.

(65) Yan, H.; Liu, B.; Du, J.; Yang, G.; Row, K. H. *Journal of Chromatography A* **2010**, *1217*, 5152-5157.

(66) Tadeo, J. L. *Analysis of Pesticides in Food and Environmental Samples*, 2008.

(67) Arsenault, J. C. *Beginner's Guide to SPE (Solid-Phase Extraction)*; Waters Corporation, 2012.

(68) Macherey-Nagel, Solid Phase Extraction (SPE), Basic principles of SPE, *2013*.

(69) Pawliszyn, J. *Applications of solid phase microextraction*; The Royal Society of Chemistry, 1999.

(70) Risticevic, S.; Vuckovic, D.; Lord, H. L.; Pawliszyn, J. In *Comprehensive Sampling and Sample Preparation*; Editor-in-Chief: Janusz, P., Ed.; Academic Press: Oxford, 2012; pp 419-460.

(71) Kataoka, H.; Lord, H. L.; Pawliszyn, J. *Journal of Chromatography A* **2000**, *880*, 35-62.

(72) Vas, G.; Vékey, K. *Journal of Mass Spectrometry* **2004**, *39*, 233-254.

(73) Augusto, F.; Luiz Pires Valente, A. *TrAC Trends in Analytical Chemistry* **2002**, *21*, 428-438.

(74) Baltussen, E.; David, F.; Sandra, P.; Janssen, H.-G.; Cramers, C. A. *Journal of Chromatography A* **1998**, *805*, 237-247.

(75) Schäfer, R. B.; Mueller, R.; Brack, W.; Wenzel, K.-D.; Streck, G.; Ruck, W.; Liess, M. *Chemosphere* **2008**, *70*, 1952-1960.

(76) Wang, W.; Meng, B.; Lu, X.; Liu, Y.; Tao, S. *Analytica Chimica Acta* **2007**, *602*, 211-222.

(77) Barriada-Pereira, M.; Concha-Graña, E.; González-Castro, M. J.; Muniategui-Lorenzo, S.; López-Mahía, P.; Prada-Rodríguez, D.; Fernández-Fernández, E. *Journal of Chromatography A* **2003**, *1008*, 115-122.

(78) Satyajit D. Sarker, Z. L., Alexander I. Gray *Natural Products Isolation, second edition*, 2006.

(79) Tadeo, J. L.; Sánchez-Brunete, C.; Albero, B.; García-Valcárcel, A. I. *Journal of Chromatography A* **2010**, *1217*, 2415-2440.
(80) Picó, Y. *TrAC Trends in Analytical Chemistry* **2013**, *43*, 84-99.
(81) Papadakis, E. N.; Vryzas, Z.; Papadopoulou-Mourkidou, E. *Journal of Chromatography A* **2006**, *1127*, 6-11.
(82) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. *Journal of AOAC International* **2003**, *86*, 412-431.
(83) Wilkowska, A.; Biziuk, M. *Food Chemistry* **2011**, *125*, 803-812.
(84) Plossl, F.; Giera, M.; Bracher, F. *Journal of Chromatography A* **2006**, *1135*, 19-26.
(85) Lesueur, C.; Gartner, M.; Mentler, A.; Fuerhacker, M. *Talanta* **2008**, *75*, 284-293.
(86) Fagerquist, C. K.; Alan, R.; Lehotay, S. J. *Analytical Chemistry* **2005**, *77*, 1473-1482.
(87) Aguilera-Luiz, M.; Vidal, J. L. M.; Romero-González, R.; Frenich, A. G. *Journal of Chromatography A* **2008**, *1205*, 10-16.
(88) Kinsella, B.; Lehotay, S. J.; Mastovska, K.; Lightfield, A. R.; Furey, A.; Danaher, M. *Analytical Chimica Acta* **2009**, *637*, 196-207.
(89) Stubbings, G.; Bigwood, T. *Analytical Chimica Acta* **2009**, *637*, 68-78.
(90) Bragança, I.; Plácido, A.; Paíga, P.; Domingues, V. F.; Delerue-Matos, C. *Science of The Total Environment* **2012**, *433*, 281-289.
(91) Correia-Sa, L.; Fernandes, V. C.; Carvalho, M.; Calhau, C.; Domingues, V. F.; Delerue-Matos, C. *Journal of Separation Science* **2012**, *35*, 1521-1530.
(92) Vera, J.; Correia-Sá, L.; Paíga, P.; Bragança, I.; C. Fernandes, V.; F. Domingues, V.; Delerue-Matos, C. *Sample Preparation* **2013**, 53-76.
(93) UCT, *2013*,https://www.chromspec.com/pdf/e/uct19.pdf.
(94) Pinto, C. G.; Laespada, M. E. F.; Martin, S. H.; Ferreira, A. M. C.; Pavon, J. L. P.; Cordero, B. M. *Talanta* **2010**, *81*, 385-391.
(95) Lehotay, S. J.; Kok, A.; de; Hiemstra, M.; Bodegraven, P. v. *Journal of AOAC International* **2005**, *88*, 595-614.
(96) AOAC Method *2013*,http://www.weber.hu/PDFs/QuEChERS/AOAC_2007_01.pdf.
(97) EN15662 In *Determination of Pesticide Residues Using GC-MS and/or LC-MS/MS Following Acetonitrile Extraction/Partitioning and Clean-up by Dispersive SPE.*, 2008.
(98) Fulton G. Kitson, B. S. L., Charles N. McEwen *Gas Chromatography and Mass Spectrometry, A Practical Guide*, 1996.
(99) Basheer, C.; Narasimhan, K.; Yin, M.; Zhao, C.; Choolani, M.; Lee, H. K. *Journal of Chromatography A* **2008**, *1186*, 358-364.
(100) Banerjee, K.; Utture, S.; Dasgupta, S.; Kandaswamy, C.; Pradhan, S.; Kulkarni, S.; Adsule, P. *Journal of Chromatography A* **2012**, *1270*, 283-295.
(101) Bolaños, P. P.; Frenich, A. G.; Vidal, J. L. M. *Journal of Chromatography A* **2007**, *1167*, 9-17.
(102) Balinova, A.; Mladenova, R.; Shtereva, D. *Journal of Chromatography A* **2007**, *1150*, 136-144.
(103) Banerjee, K.; Oulkar, D. P.; Dasgupta, S.; Patil, S. B.; Patil, S. H.; Savant, R.; Adsule, P. G. *Journal of Chromatography A* **2007**, *1173*, 98-109.
(104) Kanrar, B.; Mandal, S.; Bhattacharyya, A. *Journal of Chromatography A* **2010**, *1217*, 1926-1933.
(105) Guardia-Rubio, M.; Fernández-De Córdova, M. L.; Ayora-Cañada, M. J.; Ruiz-Medina, A. *Journal of Chromatography A* **2006**, *1108*, 231-239.
(106) Dasgupta, S.; Banerjee, K.; Patil, S. H.; Ghaste, M.; Dhumal, K. N.; Adsule, P. G. *Journal of Chromatography A* **2010**, *1217*, 3881-3889.

(107) Húšková, R.; Matisová, E.; Hrouzková, S.; Švorc, Ľ. *Journal of Chromatography A* **2009**, *1216*, 6326-6334.

(108) Robert L. Grob, E. F. B. *Modern Practice of Gas Chromatography, 4th Edition*, 2004.

(109) Alder, L.; Greulich, K.; Kempe, G.; Vieth, B. *Mass Spectrometry Reviews* **2006**, *25*, 838-865.

(110) Hubschmann, H.-J. *Handbook of CG/MS-Fundamentals and Applications*, 2009.

(111) Brossa, L.; Marcé, R. M.; Borrull, F.; Pocurull, E. *Journal of Chromatography A* **2003**, *998*, 41-50.

(112) Hoh, E.; Mastovska, K. *Journal of Chromatography A* **2008**, *1186*, 2-15.

(113) Cervera, M. I.; Medina, C.; Portolés, T.; Pitarch, E.; Beltrán, J.; Serrahima, E.; Pineda, L.; Muñoz, G.; Centrich, F.; Hernández, F. *Anal. Bioanal. Chem.* **2010**, *397*, 2873-2891.

(114) Amvrazi, E. G.; Tsiropoulos, N. G. *Journal of Chromatography A* **2009**, *1216*, 2789-2797.

(115) Liu, X.; Wang, X.; Xu, J.; Dong, F.; Song, W.; Zheng, Y. *Biomedical Chromatography* **2011**, *25*, 1081-1090.

(116) Fernandes, V. C.; Domingues, V. F.; Mateus, N.; Delerue-Matos, C. *Journal of Chromatographic Science* **2011**, *49*, 715-730.

(117) Beltran, J.; Peruga, A.; Pitarch, E.; Lopez, F. J.; Hernandez, F. *Anal. Bioanal. Chem.* **2003**, *376*, 502-511.

(118) Hunter, R. E.; Riederer, A. M.; Ryan, P. B. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 1396-1402.

(119) Lambropoulou, D. A.; Psillakis, E.; Albanis, T. A.; Kalogerakis, N. *Analytica Chimica Acta* **2004**, *516*, 205-211.

(120) Huskova, R.; Matisova, E.; Kirchner, M. *Chromatographia* **2008**, *68*, S49-S55.

(121) Vidal, J. L. M.; Arrebola, F. J.; Mateu-Sanchez, M. *Chromatographia* **2002**, *56*, 475-481.

(122) Castillo, M.; Gonzalez, C.; Miralles, A. *Anal. Bioanal. Chem.* **2011**, *400*, 1315-1328.

(123) Sherma, J. *Journal of AOAC International* **2001**, *84*, 1303-1312.

(124) Banerjee, K.; Savant, R. H.; Dasgupta, S.; Patil, S. H.; Oulkar, D. P.; Adsule, P. G. *Journal of AOAC International* **2010**, *93*, 368-379.

(125) Mandalakis, M.; Tsapakis, M.; Stephanou, E. G. *Journal of Chromatography A* **2001**, *925*, 183-196.

(126) Naert, C.; Saeger, S. D.; Van Peteghem, C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2317-2322.

(127) Yusà, V.; Pardo, O.; Pastor, A.; de la Guardia, M. *Analytica Chimica Acta* **2006**, *557*, 304-313.

(128) León, N.; Yusà, V.; Pardo, O.; Pastor, A. *Talanta* **2008**, *75*, 824-831.

(129) Üveges, M.; Abrankó, L.; Fodor, P. *Talanta* **2007**, *73*, 490-497.

(130) Banerjee, K.; Savant, R. H.; Dasgupta, S.; Patil, S. H.; Oulkar, D. P.; Adsule, P. G. *Journal of AOAC International* **2010**, *93*, 368-379.

(131) Rao, C. R. *Linear Statistical Inference and its Applications: Second Editon*, 2008.

(132) Horn, R. *Biophysical Journal* **1987**, *51*, 255-263.

(133) European Commission, DIRECTIVE 2008/105/EC *2013*,http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF.

(134) European Commission, DIRECTIVE 2000/60/EC, *2013*.

(135) European Commission, Community Strategy for Endocrine Disrupters (working document), *2013*.

(136) European Commission, 4th Report on the implementation of the "Community Strategy for Endocrine Disrupters" a range of substances suspected of interfering with the hormone systems of humans and wildlife *2013*.

(137) European Commission, *2013*,http://ec.europa.eu/environment/chemicals/endocrine/strategy/substances_en.htm#priority_list.

(138) Carreno, J.; Rivas, A.; Granada, A.; Lopez-Espinosa, M. J.; Mariscal, M.; Olea, N.; Olea-Serrano, F. *Environmental Research* **2007**, *103*, 55-61.

(139) Pestana, D.; Teixeira, D.; Faria, A.; Domingues, V.; Monteiro, R.; Calhau, C. *Environmental Toxicology* **2013**, n/a-n/a.

(140) Quirós-Alcalá, L.; Alkon, A. D.; Boyce, W. T.; Lippert, S.; Davis, N. V.; Bradman, A.; Barr, D. B.; Eskenazi, B. *NeuroToxicology* **2011**, *32*, 646-655.

(141) Domingues, V.; Cabral, M.; Alves, A.; Delerue-Matos, C. *Analytical Letters* **2009**, *42*, 706-726.

(142) Albaseer, S. S.; Rao, R. N.; Swamy, Y. V.; Mukkanti, K. *Journal of Chromatography A* **2010**, *1217*, 5537-5554.

(143) Armenta, S.; Garrigues, S.; de la Guardia, M. *Trac-Trends in Analytical Chemistry* **2008**, *27*, 497-511.

(144) Goncalves, C. M.; Esteves da Silva, J. C. G.; Alpendurada, M. F. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 6227-6235.

(145) Correia-Sá, L.; Fernandes, V. C.; Carvalho, M.; Calhau, C.; Domingues, V. F.; Delerue-Matos, C. *Journal of Separation Science* **2012**, *35*, 1521-1530.

(146) Fernandez, M. F.; Parera, J.; Arrebola, J. P.; Santa Marina, L.; Vrijheid, M.; Llop, S.; Abalos, M.; Tardon, A.; Castano, A.; Abad, E.; Olea, N. *Science of the Total Environment* **2012**, *441*, 49-56.

(147) Lino, C. M.; Noronha da Silveira, M. I. *Environmental Research* **2006**, *102*, 339-351.

(148) Ensminger, M.; Bergin, R.; Spurlock, F.; Goh, K. S. *Environmental Monitoring and Assessment* **2011**, *175*, 573-587.

(149) Feo, M. L.; Ginebreda, A.; Eljarrat, E.; Barceló, D. *Journal of Hydrology* **2010**, *393*, 156-162.

(150) Gao, J.; Liu, L.; Liu, X.; Lu, J.; Zhou, H.; Huang, S.; Wang, Z.; Spear, P. A. *Environment International* **2008**, *34*, 1097-1103.

(151) Edgar, G. J.; Barrett, N. S.; Graddon, D. J.; Last, P. R. *Biological Conservation* **2000**, *92*, 383-397.

(152) Franca, S.; Vasconcelos, R. P.; Reis-Santos, P.; Fonseca, V. F.; Costa, M. J.; Cabral, H. N. *Ecol. Indic.* **2012**, *18*, 11-19.

(153) Carabias-Martínez, R.; Rodríguez-Gonzalo, E.; Fernández-Laespada, M. E.; Calvo-Seronero, L.; Sánchez-San Román, F. J. *Water Research* **2003**, *37*, 928-938.

(154) Huntscha, S.; Singer, H. P.; McArdell, C. S.; Frank, C. E.; Hollender, J. *Journal of Chromatography A* **2012**, *1268*, 74-83.

(155) Mansilha, C.; Melo, A.; Rebelo, H.; Ferreira, I. M. P. L. V. O.; Pinho, O.; Domingues, V.; Pinho, C.; Gameiro, P. *Journal of Chromatography A* **2010**, *1217*, 6681-6691.

(156) Fernandes, V. C.; Vera, J. L.; Domingues, V. F.; Silva, L. M. S.; Mateus, N.; Delerue-Matos, C. *Journal of the American Society for Mass Spectrometry* **2012**, *23*, 2187-2197.

(157) Poole, C. F. *Journal of Chromatography A* **2007**, *1158*, 241-250.

(158) Ines Bonansea, R.; Valeria Ame, M.; Alberto Wunderlin, D. *Chemosphere* **2013**, *90*, 1860-1869.

(159) Sridhara Chary, N.; Herrera, S.; Jose Gomez, M.; Fernandez-Alba, A. R. *Anal. Bioanal. Chem.* **2012**, *404*, 1993-2006.

(160) Chau, K. W. *Chemosphere* **2006**, *64*, 1545-1549.

(161) Xue, N.; Xu, X.; Jin, Z. *Chemosphere* **2005**, *61*, 1594-1606.

(162) Caldas, S. S.; Bolzan, C. M.; Cerqueira, M. B.; Tomasini, D.; Furlong, E. B.; Fagundes, C.; Primel, E. G. *Journal of Agricultural and Food Chemistry* **2011**, *59*, 11918-11926.

(163) Rashid, A.; Nawaz, S.; Barker, H.; Ahmad, I.; Ashraf, M. *Journal of Chromatography A* **2010**, *1217*, 2933-2939.

(164) Shahpoury, P.; Hageman, K. J.; Matthaei, C. D.; Magbanua, F. S. *Environmental Pollution* **2013**, *181*, 219-225.

(165) Da, C.; Liu, G.; Tang, Q.; Liu, J. *Environmental Science: Processes & Impacts* **2013**.

(166) Kim, Y.-S.; Eun, H.; Cho, H.-S.; Kim, K.-S.; Sakamoto, T.; Watanabe, E.; Baba, K.; Katase, T. *Archives of Environmental Contamination and Toxicology* **2008**, *54*, 386-394.

(167) Syed, J. H.; Malik, R. N.; Muhammad, A. *Chemistry and Ecology* **2013**, 1-10.

(168) Gómez, S.; Gorri, D.; Irabien, Á. *Environmental Monitoring and Assessment* **2011**, *176*, 385-401.

(169) Arienzo, M.; Masuccio, A. A.; Ferrara, L. *Archives of Environmental Contamination and Toxicology* **2013**, *65*, 396-406.

(170) Sánchez-Avila, J.; Vicente, J.; Echavarri-Erasun, B.; Porte, C.; Tauler, R.; Lacorte, S. *Marine Pollution Bulletin* **2013**, *72*, 119-132.

(171) Barakat, A. O.; Mostafa, A.; Wade, T. L.; Sweet, S. T.; El Sayed, N. B. *Chemosphere* **2013**, *93*, 545-554.

(172) Vera, J.; Correia-Sá, L.; Paíga, P.; Bragança, I.; Fernandes Virgínia, C.; Domingues Valentina, F.; Delerue-Matos, C. In *Sample Preparation*, 2014; Vol. 1; pp 54.

(173) Kvicalova, M.; Doubravova, P.; Jobanek, R.; Jokesova, M.; Ocenaskova, V.; Sussenbekova, H.; Svobodova, A. *Bulletin of Environmental Contamination and Toxicology* **2012**, *89*, 21-26.

(174) Brondi, S. H. G.; Macedo, A. N.; Vicente, G. H. L.; Nogueira, A. R. A. *Bulletin of Environmental Contamination and Toxicology* **2011**, *86*, 18-22.

(175) Yang, X. B.; Ying, G. G.; Kookana, R. S. *J. Environ. Sci. Health Part B-Pestic. Contam. Agric. Wastes* **2010**, *45*, 152-161.

(176) Yurtkuran, Z.; Saygı, Y. *Bulletin of Environmental Contamination and Toxicology* **2013**, *91*, 165-170.

(177) Lazartigues, A.; Fratta, C.; Baudot, R.; Wiest, L.; Feidt, C.; Thomas, M.; Cren-Olivé, C. *Talanta* **2011**, *85*, 1500-1507.

(178) Zhang, N.; Yang, Y.; Tao, S.; Liu, Y.; Shi, K.-L. *Environmental Pollution* **2011**, *159*, 700-705.

(179) Asensio-Ramos, M.; Hernandez-Borges, J.; Ravelo-Perez, L. M.; Rodriguez-Delgado, M. A. *Anal. Bioanal. Chem.* **2010**, *396*, 2307-2319.

(180) Marques, C. P. R. R., Universidade de Aveiro, 2009.

(181) Ghanem, A.; Bados, P.; Perreau, F.; Benabdallah, R.; Plagellat, C.; Alencastro, L.; Einhorn, J. *Anal. Bioanal. Chem.* **2008**, *391*, 345-352.

(182) Devault, D. A.; Ith, C.; Merlina, G.; Lim, P.; Pinelli, E. *International Journal of Environmental Analytical Chemistry* **2010**, *90*, 311-320.

(183) Wan, M. T.; Kuo, J.-N.; McPherson, B.; Pasternak, J. *Journal of Environmental Science and Health, Part B* **2006**, *41*, 855-867.

(184) Hela, D. G.; Lambropoulou, D. A.; Konstantinou, I. K.; Albanis, T. A. *Environ Toxicol Chem* **2005**, *24*, 1548-1556.

(185) Xue, N.; Li, F.; Hou, H.; Li, B. *Environmental Toxicology and Chemistry* **2008**, *27*, 1055-1062.

(186) Woudneh, M. B.; Oros, D. R. *Journal of Chromatography A* **2006**, *1135*, 71-77.